STUDIES ON FIBRIN NETWORK STRUCTURE

by

IAN ANDREW FERGUSON

A thesis submitted for the degree of Doctor of Philosophy of the Australian National University

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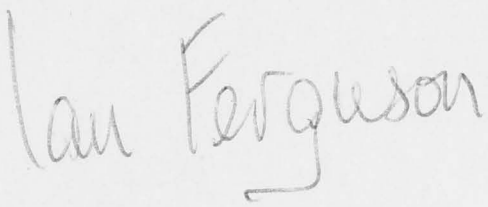
STATEMENT

Except where acknowledged, the investigations described in this thesis are my own original work



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SYNOPSIS

Fibrin networks were formed in plasma or in fibrinogen solutions under conditions which simulated those in plasma and these networks were examined with a battery of methods so as to establish whether network structure might vary *in vivo*. Studies indicate that network structure and properties in plasma changes following surgery. The structure and properties of networks in plasma were found to be sensitive to anticoagulant and clotting conditions particularly to ionic strength and pH. The post-operative increasein fibrinogen concentration appears to decrease the average thickness of fibrin fibres. The mechanical behaviour of networks in plasma differed from those made in purified fibrinogen. The structure and properties of networks formed in purified fibrinogen, like that in plasma, were also sensitive to small changes in clotting conditions. In addition, they were sensitive to many clinically and physiologically important substances, such as, heparin, protamine sulphate, glucose and glycosaminoglycans. These studies suggest that changes in the kinetics of fibrin generation and changes in the solubility of fibrin(ogen) result in changes in network structure and hence clot properties. Factor XIII mediated cross-linking did not substantially influence fibre thickness but it did reduce network permeability. It was demonstrated that cross-linking decreased the mass-length ratio determined from permeability, but not that determined from turbidity. Transmission electron micrographs showed this was due to the stabilisation of a previously undocumented network structure - the minor network. In

contrast to the assumption implicit in methods for calculating mass-length ratio of fibrin fibres it was shown that fibre thickness in fibrin gels is not homogeneous.

The gods did not reveal, from the beginning,
All things to us, but in the course of time
Through seeking we may learn and know this better.
But as for certain truth, no man has known it,
Nor shall he know it, neither of the gods
Nor yet of all things of which I speak.
For even if by chance he were to utter
The final truth, he would himself not know it:
For all is but a woven web of guesses.

Xenophanes

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ABBREVIATIONS

Å	Angstrom
βTG	Beta-thromboglobulin
γTG	Gamma-thromboglobulin
CPD	Citrate-phosphate-dextrose
CTP	Citrate-theophylline-PGE_1
DSK	Disulphide knot
FDP	Fibrin(ogen) degradation product
ΔOD/sec	Rate of turbidity increase per second
λ	Wavelength
PF_4	Platelet factor 4
PFP	Platelet free plasma
PGE_1	Prostaglandin E_1
PPP	Platelet poor plasma
SDS	Sodium lauryl sulphate
SPPS	Stable plasma protein solution
Tris	Tris (hydroxymethyl) aminomethane
μ_p	Mass-length ratio from turbidity
μ_T	Mass-length ratio from permeability

CHAPTER 1

GENERAL INTRODUCTION

1.1 FIBRINOGEN

Coagulation factor I or fibrinogen accounts for approximately 5% of the total plasma proteins. It is an inactive form of a reactive protein. When fibrinogen is activated, it forms fibrin monomers. These react with each other in a highly organized fashion to form a three dimensional network of fibres.

The structure of the fibrin network depends critically upon the fibrinogen molecule and much is known about the molecular structure of fibrinogen (reviews: Mosesson and Finlayson, 1976; Doolittle, 1977; 1980).

1.1.1 Molecular characteristics

Fibrinogen is a glycoprotein of molecular weight 340,000 daltons (McDonagh et al., 1972). It is a dimeric molecule composed of three chain pairs termed Aα, Bβ and γ of molecular weights, 64,000, 56,000, and 48,000 daltons, respectively (McDonagh et al., 1972). These chains are linked together by 29 disulfide bonds formed from 58 cysteine amino acids in each fibrinogen molecule (Henschen, 1964). The location of these disulfide linkages and the complete amino acid sequences of all the chains are known (Doolittle, 1980).

Carbohydrate moieties are attached to the penultimate amino acid of the Bβ chain, that is, asparigine, and to an

unknown site on the γ chain (Topfer-Petersen et al., 1979). There are 23 residues of hexose, 20 residues of hexosamine and 6-7 sialic acid residues per human fibrinogen molecule (Mosesson et al., 1967). These moieties are important since removal of sialic acid by neuramidinase alters the clottability of fibrinogen and the properties of the resultant clot (Chandrasekhar et al., 1962).

1.1.2 Primary structure

The primary structure of fibrinogen has been examined by cleaving human fibrinogen using cyanogen bromide into five disulfide containing fragments (Murano, 1978). These fragments, termed disulfide knots (DSK), form three groups according to their properties:

(i) N-DSK - a hydrophilic dimeric structure consisting of the amino terminal portions of all three chains. It is partially "hidden" in the intact fibrinogen structure and is contained within the plasmin derived fragment E.

(ii) Hi2-DSK - a hydrophilic single chain derived from C-terminal penultimate portion of Aα chain. It contains unusually high proportions of threonine, serine, glycine and proline and it seems to contain surface orientated hydrated structures.

(iii) HO1-DSK; HO2-DSK; HO3-DSK - are hydrophobic

structures respectively derived from: (a) all three chains, (b) the C-terminal penultimate portion of the Bβ chain and (c) the C-terminal penultimate portion of the γ chain. These structures are buried within the protein and are contained within the plasmin derived fragment D.

1.1.3 Tertiary structure

Many models of the tertiary structure of fibrinogen have been proposed (review: Lederer, 1979) varying according to the techniques used for analysis and on whether the fibrinogen examined was dehydrated or in solution. Studies on dehydrated preparations using electron microscopic examination of shadow cast, negative stained or freeze etched fibrinogen samples tend to support a "tri-nodular" model first proposed by Hall and Slayter (1959). This model depicts the fibrinogen molecule as 450 Å long with three globular domains; the end ones or D domain, being approximately 65Å in diameter whereas the central one or E domain, is 50Å in diameter. Models based on light scattering studies of fibrinogen in solution, on the other hand, support straight or bent swollen cylindrical shapes of 90Å thickness and 450Å length (Lederer and Hammel, 1975; Bachmann et al., 1975; Marguerie and Struhruman, 1976; Lederer, 1979).

Since hydrodynamic data portrays fibrinogen as a highly hydrated protein containing 4 - 6g H_2O/g protein compared

with 0.4g H_2O/g protein estimated for most globular proteins (Lederer and Hammel, 1975), the value of studying dehydrated samples is questionable.

1.2 FIBRIN FORMATION

At least three distinct steps may be identified in the process of fibrin formation:

(i) The production of monomeric fibrin by proteolytic activation of fibrinogen. Under physiological conditions this reaction is normally catalysed by thrombin (review: Doolittle, 1980).

(ii) The polymerization of monomeric fibrin by self-assembly. Monomers first associate end-to-end to form long protofibrils, two molecules in diameter and then these protofibrils associate laterally to form fibrin fibres (review: Hermans and McDonagh, 1982).

(iii) The covalent crosslinking of fibrin monomers results in a massive proteinaceous structure. This reaction is catalysed by the thrombin activated enzyme factor $XIII_a$ (review: Folk and Finlayson, 1977).

1.2.1 Thrombin and fibrin formation

Thrombin concentration increases explosively when the coagulation cascade is activated (Mann and Downing, 1976). Thrombin, a serine protease, then specifically cleaves the arginyl-glycine bond on the 16-17 position of the Aα chain and then a similar bond at the 14-15 position on the Bβ chain of fibrinogen (Blomback and Blomback, 1972; Doolittle, 1973). The peptides released are negatively charged and they are termed fibrinopeptides A and B. Their molecular weights are 1536 and 1552, respectively (Blomback et al., 1966). This thrombin mediated removal of the fibrinopeptides converts the fibrinogen molecule into fibrin monomer.

Thrombin cleaves fibrinopeptide A about five times faster than fibrinopeptide B (Martinelli and Scheraga, 1980); the second A peptide is cleaved approximately 40 fold faster than the first (Landis and Waugh, 1975). An increase in ionic strength decreases the rate of fibrinopeptide release (Blomback, 1958). Unlike fibrinopeptide A, fibrinopeptide B is released fastest when the monomer is bound to the network (Blomback et al., 1978).

1.2.2 Fibrin formation in the absence of thrombin

Fibrin can form due to the action of various snake venom enzymes such as reptilase (Blomback, 1958; Laurent

and Blomback, 1958), ancrod (Pizzo et al., 1972) and a fraction from the southern copperhead (Henzig et al., 1970), amongst others (Bilezikian et al., 1975). These thrombin-like enzymes cleave one or both fibrinopeptides. These proteases have proved useful in examining fibrinogen to fibrin conversion, showing, for example, that fibrin will polymerize even if fibrinopeptide B is not removed and that if fibrinopeptide B is removed before fibrinopeptide A, then a network will not form until an appreciable amount of fibrinopeptide A has been removed (Henzig et al., 1970; Bilezikian et al., 1975).

Studies with thrombin-like proteases suggest that the removal of specific fibrinopeptides is critical for normal fibrin formation. However, a "fibrin" network may also form if peptides which are different to those cleaved off by thrombin are removed. An aortic wall extract termed vasculokinase (Murray and Gray, 1964) and staphylocoagulase a product of certain strains of staphylococci (King et al., 1975), coagulates fibrinogen solutions by cleaving peptide bonds different to those cleaved by thrombin. Consequently, such fibrin is of different character when compared with that formed through the action of thrombin.

1.2.3 Polymerization

It is widely held that the first fibrin monomers formed by the action of thrombin bind not to themselves but to fibrinogen (Brass et al., 1976). Fibrinogen appears to mop

up free fibrin monomers and thus act like a buffer. The number of fibrinogen molecules required to solubilize one fibrin monomer varies with the experimental conditions (Landis and Waugh, 1975; Sherman, 1977). The continued action of thrombin ultimately converts fibrinogen in the fibrinogen-fibrin monomer complexes to free fibrin monomers. These monomers then self-assemble in a highly ordered fashion.

Recently the polymerization process has been extensively researched (review: Hermans and McDonagh, 1982). Studies suggest at least three different kinds of contacts when fibrin is formed. Two of these, the DE-stag and DD-long contacts, hold the fibrin monomers together in protofibrils. The DE-stag contact represents the contact between a D-domain and an E-domain of neighbouring monomers in the protofibril. (The E-domain corresponds, roughly, to fibrinolytic fragment E and the cyanogen bromide fragment N-DSK while the D-domain corresponds to fibrinolytic fragment D - see 1.1.2, 1.1.3). The DD-long contact is formed between two adjacent D-domains associated end-to-end. Relatively little is known about the third kind of contact which is the contact responsible for lateral association of protofibrils to fibres.

The polymerization process first involves the formation of protofibrils. They are formed from fibrinogen molecules activated to monomeric fibrin by the removal of fibrinopeptide A; the monomers self-associate rapidly to

form polymeric strands, two molecules in diameter. The protofibril has a helical symmetry (Hermans, 1979); the helical axis is approximately a two-fold screw axis (Hantgan et al., 1980). This arrangement of monomers accounts for the high specific volume of fibrin in fibres; fibrin fibrils and fibres contain 80% solvent and 20% protein (Carr and Hermans, 1978).

As protofibrils grow longer they associate laterally to form fibres. The tendency of the protofibrils to associate laterally is enhanced by the removal of fibrinopeptide B. Indeed, it is likely that thrombin preferentially removes fibrinopeptide B from polymerized, rather than monomeric fibrin species. During the process of lateral association long protofibrils can participate in the formation of two fibres simultaneously and this results in fibre interconnections and fibre branchpoints.

1.2.4 Crosslinking

Before crosslinking the fibrin polymer consists only of an ordered arrangement of individual fibrin monomers and it may be re-solubilized by using solutions which break hydrogen bonds. The stability of fibrin is ensured through the introduction of covalent crosslinkages between the polymerization sites on neighbouring monomers. Crosslinking welds the numerous individual monomers into one massive proteinaceous structure. This reaction is catalysed by coagulation factor $XIII_a$, a transglutaminase

activated by thrombin in the presence of Ca^{++}.

Factor $XIII_a$ introduces isopeptide bonds between the ε-amino groups of certain lysine residues on one chain and the δ-carboxyamide groups of certain glutamine residues on the neighbouring chain (review : Folk and Finlayson, 1977). In this way γ chains are dimerized and α chains are polymerized.

Factor $XIII_a$ crosslinks γ chains much faster than α chains (McDonagh et al., 1972) but the crosslinking of the α chains modulates the properties of the clot. Relative to non-crosslinked clots, the crosslinked clot is mechanically more elastic and less amenable to fibrinolysis (McDonagh et al., 1971).

1.3 FIBRIN NETWORK STRUCTURE AND PROPERTIES

The structure of the fibrin network determines the properties of the clot as a whole. The number and thickness of the fibres in the network and the extent of factor $XIII_a$ mediated crosslinking generally dictates the clot properties. But when certain fibrin fragments are incorporated into the growing fibres, the network structure becomes abnormal.

1.3.1 Normal fibrin network structure

A network of fibrin fibres is a metastable structure as

the composite fibrin fibres tend to associate laterally when closely aligned in parallel (Nelb et al., 1976). This tendency would lead to a continued increase in the mean network fibre thickness if not for the inhibition of rotational diffusion of fibres because of their length and interpenetration within the network (Hantgan and Hermans, 1979).

After the network of fibrin fibres has formed further increases in tensile strength may occur. This may result from interfibre connections or network branchpoints forming between individual fibres (Hantgan and Hermans, 1979) or from loosely packed fibres consolidating into densely packed fibres (Hantgan et al., 1980). When the network is fully developed the clot properties do not change.

Mechanical properties of fibrin network are related to the thickness and number of fibres within the network. This was first demonstrated by Ferry and Morrison (1947) who showed that in their properties, fibrin clots lie between two extreme types - elastic, fragile, non-deforming and transparent on the one hand and non-elastic, non-fragile, plastic and opaque on the other. Ferry and Morrison termed the former - "fine" clots and the latter "coarse" clots and explained the differences in terms of the amount of lateral aggregation. When end-to-end polymerization predominated, numerous fine fibres formed whereas when side-to-side aggregation was extensive, fewer but coarser fibres formed.

Numerous thin fibres restrict liquid movement more efficiently than scarcer larger fibres and consequently, fine clots resist mechanical deformation and permeation better than coarser clots. The bundling of slender fibres into massive cables enhances the tensile strength of the fibres and so coarser clots are less fragile and possess greater elastic modulus than fine clots (Kamykowski et al., 1981). The opacity of the clot reflects the thickness of the fibres as thicker fibres scatter more light.

Originally, fine clots were noted to form at high pH or ionic strength, while coarse clots formed at lower pH or ionic strength (Ferry and Morrison, 1947). Since then many factors capable of influencing fibrin network structure have become known. These include: temperature (Ferry and Morrison, 1947), fibrinogen concentration (Carr et al., 1977; Rosser et al., 1977), thrombin concentration (Rosser et al., 1977; Carr and Hermans, 1978), certain ions, especially Ca^{++} (Edsall and Lever, 1951; Abildgaard, 1964; Bang, 1967), dextran (Dhall and Bryce, 1970; Muzaffar, 1974, Carlin et al., 1976; Carr and Gabriel, 1980), heparin (Sheppard et al., 1956) and protamine sulphate (Carlin et al., 1976). The protease used to induce clotting also affects fibrin network structure. Reptilase yields finer networks (Hantgan and Hermans, 1979) and contortrix leads to coarser networks (Furlan et al., 1976) than thrombin.

1.3.2 Abnormal fibrin structure

The arrangement of fibrin monomers into fibrin strands depends on the normal conformation of the composite monomers. It is not surprising, therefore, that slow or incomplete polymerization is a characteristic of many congenital and acquired dysfibrinogenemias (Beck, 1979).

Fibrin can also be made to display abnormal network structure by simply seeding a normally polymerizing fibrinogen solution with certain fibrinogen fragments. For example, whereas normal fibrin fibres have pointed tapering ends, fibrin fibres formed in the presence of plasmin derived fibrin degradation products (FDPs - see 1.4) display a "frayed rope" appearance (Bang, 1963). Ultrastructurally, such defective fibrin networks are disorganized tangles of heterogeneous fibres and large non-fibrous aggregates. Clots made in the presence of FDPs are slow to form, mechanically weak and easily solubilized by plasmin (Hermans and McDonagh, 1982).

Fibrin(ogen) derivatives inhibit normal polymerization by binding to polymerizing fibrin thus preventing lengthening and thickening of the fibre (Bang, 1967; Hermans and McDonagh, 1982). The early products of plasmin digested fibrinogen, such as fragment X, may be clotted by thrombin and incorporated into the growing fibre. Further normal linkage at these sites is impossible. More extensive plasmin digests yield fragment E, a competitive inhibitor

of thrombin (Budzynski et al., 1979), and fragments D and DD, the most potent inhibitors of fibrin assembly (Dray-Attali and Larrieu, 1977). The (DD)E complex does not exhibit anticoagulant effects (Budzynski et al., 1979).

1.4 FIBRINOLYSIS

Fibrin forms a temporary structure in vivo. Its removal is accomplished by proteases from leukocytes (Plow and Edgington, 1975), by phagocytosis or by the protease, plasmin (review : Gaffney, 1978). It is believed that plasmin mediates the bulk of fibrinolysis in vivo.

1.4.1 Lysis by plasmin

Plasmin is formed when its inactive precursor, plasminogen, is activated following stimulation of either the intrinsic or extrinsic fibrinolytic pathways (Wiman, 1978). Plasmin cleaves fibrin at specific peptide bonds yielding FDPs with unique properties.

When plasmin is bound to fibrin it cleaves the α, β and γ chains in an ordered fashion. It is now widely accepted that plasmin degrades both fibrinogen and fibrin according to an assymmetric scheme first proposed by Marder and co-workers (Marder et al., 1969). According to this scheme, plasmin systematically prunes the fibrin(ogen) molecule to fragment X, then to fragment Y and finally into one fragment E and two fragment Ds (review : Mihalyi, 1980).

Plasmin first removes a single polypeptide of molecular weight 40,000 to 50,000 from the COOH terminal end of the chain (Furlan and Beck, 1972; Mills and Karpatkin, 1972). Although, the γ chain is initially left intact, a NH_2 terminal fragment is cleaved off the β chain (Takagi and Doolittle, 1975). Fibrinogen degraded to this stage is termed fragment X. It is slightly coagulable by the action of thrombin as it still possesses fibrinopeptides A but not B (Budzynski et al., 1974). Fragment X has a molecular weight in the region of 250,000 and it occasionally complexes with itself in the absence of thrombin (Marder and Shulman, 1969). This characteristic is lost after fragment Y is formed.

When fragment X is degraded into fragment Y, (molecular weight about 155,000) a fragment containing the D domain, fragment D, is released. Fragment D, molecular weight 94,500, is not degraded further and it retains the γ-polymerization site.

Plasmin cleaves fragment Y into another fragment D and fragment E. Fragment E, molecular weight 55,000, contains the N-DSK and, like fragment D, it is not degraded further by plasmin.

Fibrinolysis of crosslinked fibrin differs from that of non-crosslinked fibrin in that the covalent bonds introduced by factor $XIII_a$ continue to link some of the fragments. Thus, when crosslinked fibrin is extensively

digested by plasmin a double D or DD dimer species is formed (Gaffney and Brasher, 1973). The D fragments are dimerized since the γ- polymerization sites are cross-linked (Gaffney and Brasher, 1973). The D dimer is commonly found to be non-covalently bonded to fragment E (Hudry-Clergeon et al., 1974; Gaffney et al., 1975) and this DD(E) complex is the predominant species resulting from physiological digestion of crosslinked fibrin (Plow et al., 1977; Gaffney and Joe, 1979).

Crosslinking also affects the rate of lysis. Crosslinked fibrin is more resistant to lysis than non-crosslinked fibrin (McDonagh et al., 1971) and this resistance to lysis is attributed to the presence of α polymers; γ-dimers have little influence on lysis (Gaffney and Whitaker, 1979).

1.5 PHYSIOLOGICAL ROLE OF FIBRIN

Fibrin serves a variety of functions in vivo. It acts as a molecular sieve in selectively trapping certain substances within its meshwork. The soluble products released during fibrin formation and lysis attract phagocytic cells and stimulate vascular changes. In addition, the fibrin mesh provides a temporary structural support over which cells migrate while they grow and lay down connective tissue. In these and other ways, fibrin participates in the haemostatic mechanism, the inflammatory response, the wound repair process as well as in pregnancy and malignancy.

1.5.1 Fibrinopeptides and FDPs

As has been already reviewed, activation of fibrinogen results in the release of fibrinopeptides concommitant with fibrin formation. It appears that the fibrinopeptides regulate and co-ordinate the inflammatory response. Fibrinopeptide B is chemotactic for neutrophils and monocytes (Kay et al., 1974). The fibrinopeptides are vasoconstrictive (Colman et al., 1967; Osbahr, 1975) and potentiate bradykinin induced smooth muscle contraction (Osbahr et al., 1964; Blomback et al., 1969).

During the early stages in wound repair, fibrin is lysed into FPDs which affect haemostasis. It is known that FDPs inhibit coagulation by interfering with platelet aggregation (Larrieu, 1981) and normal fibrin polymerization (Bang, 1963; 1967). FDPs affect the inflammatory response by enhancing the permeability of capillaries (Malofiejew, 1971; Barhart et al., 1971) and by attracting leukocytes (Stecher and Sorkin, 1972; Richardson et al., 1976). FDPs also modulate the vasoactive effects of bradykinin, oxytocin, histamine, epinephrine and serotonin (Malofiejew, 1971). Furtermore, at least in the rat, FDPs potentiate the hypotensive effects of bradykinin and the hypertensive effect of angiotensin (Malofiejew, 1971).

1.5.2 Physiological roles of fibrin

Characteristically fibrin normally forms only at sites of trauma or in regions of cell migration and tissue remodelling. This is not surprising because fibrin is a structural protein which is relatively rapidly formed and resolubilized. Consequently, fibrin serves vital roles in haemostasis, inflammation (Riddle and Barhart, 1964; Charkabarti, 1978), pregnancy (Bonnar, 1978) and in malignancy (reviews: Donati and Poggi, 1980; Edgington, 1980).

The participation of fibrin in haemostasis is well known. Morphologically, fibrin appears to reinforce and consolidate the platelet aggregates in the haemostatic plug (Wester et al., 1978). In both haemostatic plugs (Sixma and Wester, 1977) and thrombi (Gottlob, 1975; Hattori et al., 1978; Hisano, 1978) fibrin fibres found associated with platelet aggregation are much thicker than those found ensnaring erythrocytes.

Fibrin and platelets interact intimately during haemostasis. The plasmatic atmosphere of platelets is the focus of thrombin production (Walsh, 1977) and fibrin forms first on the surface of aggregating platelets (Hattori et al., 1978). Fibrinogen is essential for platelet aggregation (Caen et al., 1977) and indeed, polymerizing fibrin aggregates platelets (Niewiarowski et al., 1977). Platelets retract and compact the fibrin network when it

has formed and the internal resistance of the fibrin network to deformation strongly influences platelet mediated clot retraction (Cohen et al., 1975).

Fibrin can also form in the absence of platelets following an increase in the permeability of blood vessel walls and this is seen in inflammation. The inflammatory response allows the escape of large molecules including fibrinogen into the extracellular fluid. Coagulation of oedema fluid results in an extravascular fibrin network (Walter and Israel, 1974).

The mechanism of fibrin formation in the extravascular space has not been extensively examined. Coagulation may be initiated following release of tissue activator and subsequent activation of the extrinsic section of the coagulation cascade. Indeed, in delayed type hypersensitivity, macrophages release tissue activator-like procoagulant (as do some tumour cell lines - review: Donati and Poggi, 1980) which results in an extravascular fibrin mesh which temporarily imprisons the macrophage (Hopper et al., 1981).

Fibrin is often found associated with rapidly growing tissue. It is deposited at the invading periphery of malignant neoplasms (review: Donati and Poggi, 1980; Edgington, 1980) and is a prominent feature of the uteroplacental vasculature (Bonnar, 1978). In these areas there is both rapid fibrin deposition and lysis.

Fibrin is generally thought to facilitate response to injury in a number of ways. In the initial stages, fibrin assists by forming a union between severed tissues (Walter and Israel, 1974) and this limits the exudative process and provides a haemostatic barrier (Astrup, 1968). This fibrin mesh also acts as a barrier to bacterial invasion and probably facilitates phagocytosis of organisms by the invading leukocytes (Walter and Israel, 1974). Likewise, the fibrin mesh which surrounds certain malignant tumours has been suggested to provide a protective barrier against cellular attack by activated macrophages and various types of cytolytic cells involved in the cellular immune response (Dvorak et al., 1979).

In addition to providing a barrier which defines and localizes the injured or rapidly growing region, fibrin also stimulates and supports the growth of certain tissues. Fibroblasts (Nozawa, 1977; Pohl and Christophers, 1979) and neutrophils (Riddle and Barnhart, 1964), are among the first cells which migrate or grow into the fibrin rich region, and these cells alter morphology when they come into contact with fibrin in cell culture; they adhere to and grow along the fibrin fibres. The growth of fibroblasts (Pohl et al., 1979), as well as certain tumours (O'Meara, 1968), is also stimulated by fibrin. In fact, fibrin has been suggested to provide a source of amino acids for tumour cell growth (Zanker et al., 1980).

Similarly, fibrin network is thought to stimulate

(Florey, 1970) and structurally support (Banerjee and Glynn, 1960; Astrup, 1968) the ingrowth of capillary buds. Bonnar (1978) suggests that fibrin maintains the integrity of the maternal and foetal circulations by temporarily supporting the rapidly growing vessels in the placenta deprived of their musculo-elastic content. In malignancy the fibrin meshwork is thought to facilitate vascularization and hence, rapid growth of tumours (O'Meara, 1968).

Fibrin is steadily formed and lysed in the plasmatic zone in close proximity to the blood vessel wall (Copley, 1979). Endothelial fibrin lining is critical to the development of atheroma according to two recently postulated theories of atherogenesis (Copley, 1979; Kandish, 1979).

A bleeding tendency or defective wound healing is associated with congenital dysfibrinogenemias which is associated with abnormal fibrin structure and abnormal thromboelastic patterns (Crum, 1977). It seems possible that the network structure of fibrin is critical to normal function in haemostasis, inflammation, wound healing and pregnancy.

Pure fibrin networks apparently do not form <u>in vivo</u>. It has been calculated that the mass of an average plasma clot is about 95% fibrin, 4% fibronectin and 1% α_2-antiplasmin (Mosher, 1980). Fibronectin, a physiologically

important glycoprotein involved in events such as cell attachment and growth (Mosher, 1980), is crosslinked to the α chain of fibrin by factor $XIII_a$ (Mosher, 1975; 1976). α_2-antiplasmin is also crosslinked to the α chain of fibrin by factor $XIII_a$ (Sakata and Aoki, 1980). It is the most potent physiological inhibitor of plasmin (Wiman and Collen, 1978). These network constituents are thought to influence inflammation and wound healing (Hermans and McDonagh, 1982).

Haemostasis, inflammation and wound healing may also be affected by components adsorbed onto fibrin fibres, for example, plasmin(ogen) and tissue plasminogen activator (Hisano, 1978). In fact, the rate and extent of plasmin degradation *in vivo* is controlled, at least partly, by both non-covalent and covalent interactions of the fibrinolytic components with fibrin (Hermans and McDonagh, 1982). Fibrin acts as an effective antithrombin as it strongly adsorbs free thrombin (Liu *et al.*, 1979). Many other plasma proteins such as the immunoglobulins, IgG, IgA and IgM, are also trapped within fibrin clots (Regeoczi, 1968). Thus, it appears that fibrin gels behave like the gels used in affinity chromatography and this behaviour probably also influences the physiological function of fibrin.

As an overview it may be seen that fibrin plays important roles in a number of reparative mechanisms *in vivo* and that the function of fibrin in these various roles may be dependent on its structure.

1.6 PROBLEMS FOR STUDY

It is apparent that fibrin is important in a variety of ways in several reparative and pathological processes. However, a great deal of research in fibrin formation has concentrated on kinetics of fibrin polymerization. Fibrin network has not received much attention other than in some early studies which showed that fibrin fibre thickness within networks may be varied by manipulating conditions of clotting *in vitro*. These early studies were undertaken in a pharmacological context and whether fibrin networks formed under physiological conditions are open to modification by subtle changes in clotting conditions remains unexamined. To a great extent research in this area has remained hampered by the lack of suitable methods for examining fibrin networks. However, more recently a number of techniques have become available. These techniques are based on one of a number of known characteristics of fibrin networks. Thus, the permeability method (Carr *et al*., 1977) relies on perfusion characteristics or pore size (Blomback and Okado, 1982b) of the network; the turbidity method relies on differential light scattering properties by fibres of different thicknesses (Carr and Hermans, 1978).

Detailed quantification in a network requires that for a given network information should be available not only on the mean pore size, fibre thickness, and fibre density but also some idea should be obtained of the range of fibrin

diameters and pore sizes, the degree of partial transformation of fibrinogen to fibrin as well as the tensile behaviour of the network. Early studies by Ferry and Morrison (1947) attempted to relate all the various characteristics of networks to fibre thickness. However, since these early studies the introduction of new techniques theoretically allows a re-examination of several aspects of the physiology of fibrin network. This is the major concern of the studies described in this thesis which examines problems in three main areas:

1.6.1 Methodological problems

Several methods for measuring network characteristics have been introduced in recent years. The methods are based on widely different theoretical considerations. A detailed comparison of methods is lacking. This requires to be undertaken to highlight not only reproducibility of the various techniques but also to establish relationships between the various properties within a single network e.g., fibre thickness and porosity. In addition, a detailed examination is needed to establish the relationship between the various methods, based as they are on distinct characteristics of the network.

Many of the recently introduced techniques purport to measure a single value of mass-length ratio (which is proportional to fibre thickness). These methods are all based on the assumption that the fibre thickness in fibrin

networks has a uniform and homogeneous size. This fundamental assumption needs a full and thorough investigation.

In early studies (Ferry and Morrison, 1947; Dhall and Bryce, 1970; Dhall et al., 1976), semi-quantitative methods such as opacity ratio and compaction were used to characterize fibrin networks. The methods are based on theoretical reasoning but experimental verification for these techniques is lacking and needs examination.

1.6.2 Problems in the regulation of fibrin network

In the past, experimental studies on the structure and properties of fibrin networks have been primarily undertaken to examine the kinetics of fibrin polymerization. These studies have demonstrated that fibrin networks are sensitive to extreme changes in clotting conditions but it is not known whether networks are similarly sensitive to changes in clotting conditions within the patho-physiological range. Nor is it known whether structural changes are influenced by factor $XIII_a$ mediated crosslinking. These areas need examination.

It has been shown by Ferry and Morrison in 1947 that fibrin network properties may be modified by several substances. Since then isolated studies have shown that defective polymerization leads to a network which is ineffective for haemostasis. However, systematic studies

on whether fibrin networks are affected by the presence of substances used clinically or encountered within the blood are lacking. Not only should this area be examined fully but mechanisms which underlie any modification should be examined, hopefully to provide further therapeutic approaches to the management of problems in haemostasis and thrombosis.

1.6.3 Problems of studying fibrin networks in a clinical context

Several methods have been used in the past to examine networks formed in fibrinogen solutions *in vitro* but their applicability to sequential study of networks formed in plasma in a clinical setting is unclear. When plasma is examined it is normally anticoagulated. As a first step, the effect of various anticoagulants on fibrin network needs examination. Networks in plasma contain several substances in addition to those formed from purified fibrinogen, for example, fibronectin, α_2-antiplasmin and plasminogen. It is not known whether fibrin networks formed in plasma are different from those formed from purified fibrinogen at the same concentration of the latter. This area requires elucidation.

Abnormal structure and properties of fibrin networks in plasma have long been a clinical problem encountered in a variety of coagulopathies. However, to date comparative studies on fibrin network structure and properties have not

been reported in these pathological situations. This stems, in part, from a lack of sensitive and suitable methods. There is a real need at a clinical level for methods which would allow sequential studies on fibrin network. The available techniques need to be evaluated at this level and gauged for their suitability. Once methods have been shown to be applicable to clinical studies a variety of clinical and physiological situations need examination to establish the role of fibrin network in health and disease.

CHAPTER 2

MATERIALS AND METHODS

General methods used throughout this thesis and described in this Chapter. Special methods and techniques are described in appropriate chapters.

2.1 MATERIALS

2.1.1 General

Disposable plastic test tubes were used wherever possible. All glassware was cleaned by soaking in Pyroneg solution (Diversey, Sydney), then scouring with a bristle brush, followed by extensive tap water rinsing and at least two rinses with distilled water. Glassware was then oven dried at about 80°C. When glass was used (e.g., pasteur pipettes), it was pretreated with siliconizing solution (Siliclad, Clay Adams, U.S.A).

2.1.2 Fibrinogen

4gm of human fibrinogen (Kabi, grade L, Stockholm) was dissolved in 120ml of 0.895% NaCl and dialysed at 4°C against 4 litres of 0.895% NaCl for 20 hours (3 changes). Following dialysis, fibrinogen solutions were centrifuged for 1 hour at 50,000g at 4°C to clarify them. 0.5ml aliquots of the supernatant were kept frozen in plastic tubes at -20°C for periods less than two weeks and at -77°C for longer periods. Fibrinogen concentration was measured using the assay of Ratnoff and Menzie (1951).

2.1.3 Radiolabelled fibrinogen

0.8mg fibrinogen labelled with ^{125}I (Amersham, U.K.) (approximately 110uCi) was dissolved in 500µl distilled water. Duplicate 10µl samples were counted for 3 minutes in a 1280 Ultragamma counter (LKB, Wallac).

2.1.4 Thrombin

Bovine thrombin (Parke, Davis and Co., Detroit) was dissolved in distilled water to a concentration of 150U/ml and 0.3ml aliquots were kept frozen in plastic tubes at -20°C for less than two weeks or at -77°C for longer periods.

2.1.5 Tris-saline buffer

50mM Tris-HCl buffered saline (pH 7.35 and ionic strength 0.153) was used throughout the experiments to wash or perfuse clots.

2.1.6 Other reagents

Except where otherwise stated, all other chemical reagents were of analytical reagent grade and obtained from B.D.H. (Melbourne) or Sigma (St. Louis, U.S.A.). Distilled water was used throughout for making solutions.

2.2 METHODS

Throughout studies in this thesis special care was taken to form clots under conditions which mimicked those in plasma. Fibrinogen concentration was usually 3.3 mg/ml, the mean plasma concentration in health (Diem and Leutner, 1970). The final thrombin concentration was usually 0.15 U/ml, which is the concentration of thrombin present at the moment clotting of freshly drawn native blood (Shuman and Majerus, 1976).

2.2.1 Fibrin networks

Fibrin networks were formed by adding thrombin to fibrinogen dissolved in 50mM Tris-HCl-saline. Preliminary experiments showed that 50mM Tris-HCl could buffer fibrinogen solutions adequately without influencing the opacity ratio. Fibrinogen solutions also contained either 1.20mM $CaCl_2$ and 0.82mM $MgCl_2$ or 2.02mM $MgCl_2$ or neither divalent cation. Unless otherwise stated, NaCl was added to adjust the ionic strength to 0.153. The pH of the fibrinogen solution was 7.35.

Ionic strengths were adjusted with NaCl and calculated on basis of the molarity of electrolytes. Activity coefficients, degree of calcium binding to protein and contributions of added macromolecular substances were not taken into account.

Clotting was initiated at room temperature by mixing 40µl of thrombin in 960µl of fibrinogen solution. Solutions were left to clot for 30 minutes before being examined. Thrombin and fibrinogen solutions were used within 30 minutes of thawing. Throughout experiments thrombin was kept on ice.

2.2.2 Turbidimetric studies

Clots were formed in 1ml quartz cuvettes of 1cm pathlength. Clots were examined in a Unicam SP1800 spectrophotometer using unclotted fibrinogen solution as a reference.

2.2.2.1 Fibrin network development

The development of fibrin network was followed using a method adapted from Hantgan and Hermans (1979). The increase in optical density at 350nm due to network development was recorded with a Unicam AR25 linear recorder coupled to the spectrophotometer. A tangent was drawn through the 0.25 absorbance unit point on the absorbance tracing and extrapolated to 0 intensity. The slope of this line defined the rate of turbidity increase (ΔOD/sec). The distance from the origin to the intercept of this line and 0 intensity defined the lag time. The lag time is the period when fibrin is being formed prior to fibre growth.

2.2.2.2 Opacity ratio

The opacity ratio of fibrin networks has been used as a measure of the thickness of fibres in a network (Ferry and Morrison, 1947; Dhall and Bryce, 1970; Muzaffar et al., 1972; Dhall et al., 1976). This is based on the following theoretical considerations (Dhall and Bryce, 1970):

The Rayliegh-Gans equation relates turbidity with size of suspended spherical particles according to the following equation:

$$\frac{T}{C} = \frac{4\pi\kappa^3}{\lambda} \frac{d}{d_2}12 \frac{(m^2-1)}{(m^2+2)} \times 10^{-2} \quad \ldots 1$$

where T is turbidity, C is concentration of particles, κ is $\pi D/\lambda$, D is the diameter of particles, λ is the wavelength of incident light, d_{12} the density of suspension with additives, d_2 is the density of suspension without additives, m is n_1/n_o where n_1 is the refractive index of fibrin and n_o that of the medium.

For a given solution, d_{12}, d_2, n_1 and n_o, like π and 10^{-2}, can be regarded as constants (Muzaffar et al., 1972) and so the equation may be simplified to:

$$\frac{T}{C} = K \frac{D^3}{\lambda^4} \quad \ldots 2$$

For turbidities T_1 and T_2 of the same suspension at two different wavelengths λ_1 and λ_2, the equation may be written as:

$$\frac{T_1}{T_2} = \frac{(\lambda_2)^4}{(\lambda_1)^4} \quad \ldots 3$$

If the size of particles is small as compared with the wavelength of incident light, then the ratio of turbidities, the opacity ratio, may be predicted from the known wavelengths. For λ_1 = 608nm and λ_2 = 350nm the theoretical turbidity ratio of a solution containing particles smaller than the wavelength of incident light should be 0.1099. Departures from this ratio depend upon different degrees of scattering of transmitted light at the two wavelengths by the inhomogeneities. The larger the inhomogenieties formed, the larger the opacity ratio.

It should be noted that opacity ratio has been defined in a different way in this thesis to the definition in the literature. In this thesis opacity of the fibrin network at 608nm was divided by that at 350nm. Thus, that opacity ratio is directly related to the size of inhomogeneities in the network. In previous studies, (Dhall and Bryce, 1970; Muzaffar et al., 1972; Dhall et al., 1976), the opacity ratio was defined as the ratio of turbidity of fibrin network at 350nm divided by that at 608nm so that opacity ratio related inversely to the size of the inhomogeneities.

2.2.2.3 Mass-length ratio using one wavelength

The average mass-length ratio of fibrin fibres can be calculated from measurements of the turbidity at a single wavelength. Turbidity (T) is given by the equation (Carr and Hermans, 1978):

$$T = 44\pi Kc\lambda\mu/15n \qquad \ldots 4$$

where c is the concentration of fibrin fibres, λ is the wavelength, n is the refractive index of the solution and μ is the mass length ratio of the fibre in daltons/cm. The constant K is given by:

$$K = 2\pi^2 n^2 (dn/dc)^2 / N\lambda^4 \qquad \ldots 5$$

where dn/dc is the specific refractive index increment of the solute in the solvent and N is Avogadro's number. This equation is valid provided the diameter of the fibrin fibres is small compared with the wavelength of incident light.

To calculate the average mass-length ratio of fibrin fibres from measurements of turbidity at 608 nm (μ_T) in purified fibrin clots, the values of n and dn/dc used were 1.336 and 1.001, respectively (Appendix 1). Thus, from equations 4 and 5:

$$\frac{T\lambda^3}{\mu c} = \frac{44}{15} 2\pi^3 n (dn/dc)^2 / N$$

$$= 1.48 \times 10^{-23} \qquad \ldots 6$$

$$\mu_T = \frac{T\lambda^3}{1.48c} \times 10^{23}$$

$$= 15.186 \times \frac{T}{c} \times 10^{12} \text{ daltons/cm} \qquad \ldots 7$$

where c is in mg/ml and where T = 2.303 x optical density.

To calculate μ_T in plasma clots different refractive indices were used (Appendix 1).

Except where otherwise stated, μ_T was preferentially calculated from turbidity at 608 nm as it correlated linearly with μ_T from turbidities at a range of wavelengths (Appendix 2).

2.2.2.4 Mass-length ratio from a range of wavelengths

Hantgan and Hermans (1979) showed that:

$$\frac{44\pi Kc\lambda}{15Tn} = (1 + 184\pi^2\rho^2n^2/77\lambda^2 \ldots)/\mu \qquad \ldots 8$$

and that for cylindrical fibres of radius r,

$$\rho^2 = \frac{r^2}{2} \qquad \ldots 9$$

where ρ is the radius of gyration and where equation 8 is derived, in part, from equations 4 and 5. Equation 8 may be expressed in the linear form Y = A+BX by using equations 5 and 9 and by neglecting higher order terms:

$$\frac{c}{\lambda^3 T}\left[\frac{88\pi^3 n(dn/dc)^2}{15n}\right] = \frac{1}{\mu} + \frac{r^2}{\mu}\left[\frac{184\pi^2n^2}{154\lambda}\right] \qquad \ldots 10$$

A plot of C/λ^3T vs $1/\lambda^2$ can be used to calculate mass-length ratio, μ_T, and the square of fibrin fibre radius from the intercept and initial slope, respectively. Thus, using values in equation 6 it is found that:

$$\mu_T = 10^{13}/1.48A \quad \text{daltons/cm} \qquad \ldots 11$$

$$r^2 = 1/20.9(A/B) \quad \text{Å} \qquad \ldots 12$$

2.2.3 Mass-length ratio from permeability

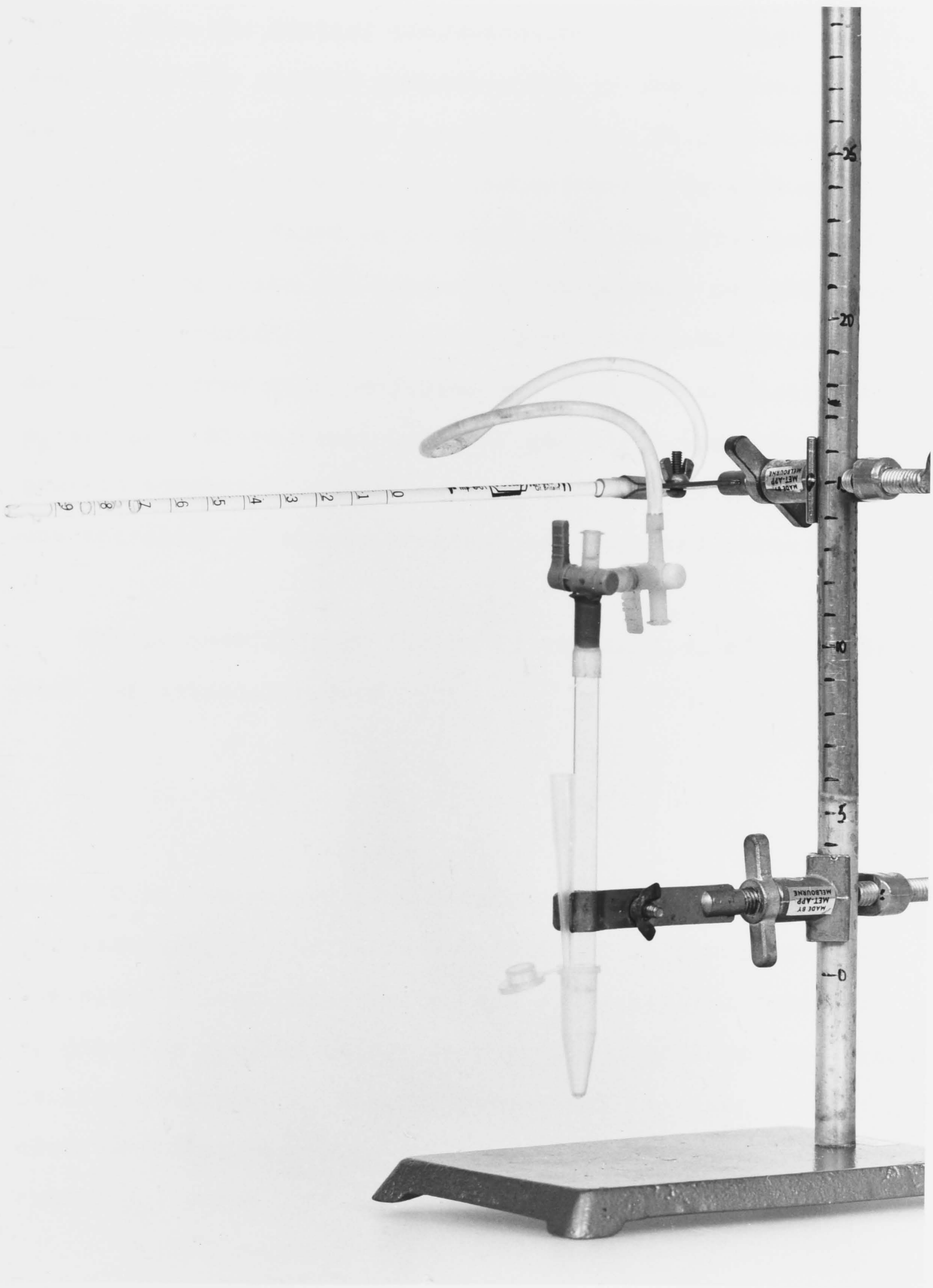
This technique is taken from Carr et al. (1977). Fibrin networks were formed in pre-etched glass tubes (24 hours in 1% HF) cut from 2ml pipettes. These tubes were approximately 10cm x 0.4cm internal diameter; the individual internal dimensions of each tube were accurately measured.

Clots were formed in tubes with one end covered by parafilm. Before testing the parafilm was removed, clot length recorded with a vernier caliper and a micro-centrifuge tube fitted to collect perfusate. The tube containing the clot was then attached to a horizontally mounted 1ml pipette on a retort stand using plastic tubing (Figure 2.1). Clots were perfused with Tris-saline.

The pressure head was increased by 5cm each three minutes until a pressure of 15cm of H_2O was reached. Clots were first perfused for 20 minutes. The transit time of an accurately measured volume of perfusate (in the region of 0.5ml) was then recorded.

The first 0.5ml of clot perfusate was collected and

Figure 2.1 The apparatus used for perfusing fibrin networks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radioactivity counted or protein concentration measured. In purified fibrinogen solutions, protein concentration was determined with a standard curve using the absorbance at 282nm of 400µl of clot perfusate and 600µl of 40% urea/0.2M NaOH. From the initial concentration of fibrinogen was subtracted the protein concentration in the perfusate to give the network protein concentration. In preliminary studies, the network protein concentration determined by this method was found to be similar to that calculated from centrifuging clots and measuring the protein concentration in the supernatant. The network protein concentration determined from clot perfusion was used in calculating both μ_p and μ_T. Where clots were not perfused, the network protein concentration was determined from the protein concentration in the supernatant of compacted clots.

The permeability or the Darcy constant, τ, of the fibrin clot was calculated from:

$$\tau = \frac{Q\,h\eta}{F\,t\,p} \qquad \ldots 13$$

where Q is the volume flow through the clot in time t; η is the viscosity of the perfusion fluid; h is the length of the clot; F its cross-section and p the applied pressure. By assuming a value of 10 (Carr et al., 1977) for the fibre packing constant, k, the Darcy constant and fibrin concentration, c, can be used to calculate the mass-length ratio, μ_p, using:

$$\mu_p = \tau k c \pi / 4 \qquad \ldots 14$$

2.2.4 Fibrin network compaction

The measurement of fibrin network compaction is based on a method of Dhall et al. (1976). Fibrin network is formed in a 1.5ml Eppendorf microcentrifuge tube presprayed with lecithin based aerosol (Spray and Cook, Woolworths) in order to render the surface non-adhering. Clots are then centrifuged at 8000g for 45 seconds in a Eppendorf microcentrifuge, Model 3200. The volume of fluid expelled from the network is measured with a 1ml syringe and expressed as a percentage of initial volume.

2.2.5 Scanning electron microscopy

200µl of clotting fibrinogen solution was pipetted onto 12mm diameter coverslips and left undisturbed for 30 minutes. Clots so formed were then immersed for 30 minutes in 2.5% glutaraldehyde in Tris-saline buffer. Following fixing, they were washed with three changes of Tris-saline and distilled water and then either freeze dried or critical point dried. For freeze drying, gels were snap frozen in liquid nitrogen and freeze dried in a Dynavac tissue freeze dryer, Model L140M for 20 hours. For critical point drying, clots were dehydrated with a graded series of acetone followed by critical point drying using CO_2 in a Bomar SPC - 1500 critical point dryer. Dehydrated clots were coated with approximately 300nm of

gold in a Dynavac vacuum coating unit and examined with a Cambridge S 1800 scanning electron microscope at a variety of magnifications.

2.2.6 Transmission electron microscopy

Fibrin gels were formed on carbon coated, parlodion film reinforced 400 mesh grids. Thrombin was added to fibrinogen solution and a drop of the clotting solution immediately placed on the grid. Most of the solution was then absorbed with filter paper so that only a very thin film remained on the grid. As that gelling film tended to dry rapidly, a final thrombin concentration of 1.5U/ml was used. The gel was left to develop for 4 minutes and then flooded with saturated aqueous uranyl acetate for 15 minutes. Grids were washed twice with tris-saline then with distilled water and finally air dried. Grids were examined under a Phillips EM301 microscope fitted with a goniometer stage. An accelerating voltage of 40 kV and an objective aperture of 40 μm were used to view the specimen.

2.2.7 Statistical analysis

Analyses were performed with the aid of a Hewlett Packard 9815A bench top calculator and supplied statistical packages. Plots were made with a Hewlett Packard 7225A plotter. Except where otherwise stated, levels of significance p(t) were calculated using students test for 2

tailed distributions. The F probabilities - p(F) - were calculated F values derived from data fitted to a linear regression model.

SECTION I

STUDIES ON METHODS USED FOR EXAMINING FIBRIN NETWORKS

CHAPTER 3

METHODS: REPRODUCIBILITY AND TECHNICAL LIMITATIONS

3.1 INTRODUCTION

A number of methods have been developed and used to study fibrin networks. Methods have been based on transmission (Tesch et al., 1979) and scanning electron microscopy (Carlin et al., 1976), light scattering and turbidimetric techniques (Dhall and Bryce, 1970; Carr et al., 1977), permeability (Rosser et al., 1977; Carr et al., 1977), compaction (Dhall et al., 1976) and viscoelastic properties (review: McIntire, 1980). In this Chapter reproducibility and technical limitations of some of these methods, particularly of those used in studies on fibrin networks described in this thesis will be considered.

3.2 MATERIALS AND METHODS

Networks were examined using all the methods described in Chapter 2. The networks were formed on different days using different batches of fibrinogen and thrombin. The coefficient of variation and the reproducibility of the methods were compared.

3.3 RESULTS

3.3.1 Reproducibility of measurements

Table 3.1 shows that for any one method, the day to day variation in the measurements is of similar order of

magnitude to the reproducibility on any one day. However, although measurements could be reproducibly made from single batches of fibrinogen and thrombin, the measurements in networks formed using different batches of fibrinogen and thrombin were significantly different. Neverthless, identical treatments (e.g., changes in ionic strength) induced a similar magnitude and direction of changes in the measurements in different batches of fibrinogen and thrombin (Figure 3.1).

The coefficient of variation in the turbidimetric techniques, in general, is lower than that in the mechanical techniques (Table 3.1). The opacity ratio and μ_T are highly reproducible. The data from compaction and permeation, on the other hand, are subject to larger intraexperimental variation.

3.3.2 Technical limitations of the methods

3.3.2.1 Turbidimetric studies

Turbidity and ΔOD/min could be easily measured. At physiological fibrin concentrations turbidity at 608 nm was directly proportional to λ^{-3} (Appendix 2) so μ_T could be calculated from turbidity at 608 nm. For good theoretical reasons (Carr and Hermans, 1978), this linear relationship may breakdown at relatively high fibrin concentrations.

3.3.2.2 Compaction

Compaction method depends upon accurate measurement of the expelled volume. In the system used, very small volumes of expelled fluid could not be measured accurately. The lower limit of accuracy of this method was set at 20 µl, that is, 2% compaction.

3.3.2.3 Perfusion studies

The critical variables in determining mass-length ratio in perfusion studies are the flow rate and network protein concentration. Initially, the perfusion fluid was coloured with Azure A to facilitate the observation of bolus movement through the clot (Carr et al., 1977). With experience the addition of dye was omitted because non-uniform flow could be easily detected from non-constant and greatly enhanced flow rates often associated with collapse of the clot. A minimum of three identically prepared networks were always perfused together. Following an initial stabilizing period (usually less than 20 minutes), the flow rate through clots remained constant (Figure 3.2). The protein concentration could be determined with as little as 0.1 ml of perfusate. However, because of slow flow rate in some very fine networks, evaporation significantly reduced the perfusate volume. Consequently, calculations of μ_p of very fine clots may be subject to small but acceptable errors.

3.3.2.4 Transmission electron microscopy

A fine and coarse fibrin network are shown in Figure 3.3. It may be seen that the fibres in the coarse network are, on the average, thicker than those in the finer network. Note also that within both networks the thickness of fibres is not uniform.

As fully described in Chapter 4, the network structure may be examined quantitatively in transmission electron micrographs using morphometric analysis. However, this proved time consuming but it was particularly suited to examining the internal structure of fibrin networks.

3.3.2.5 Scanning electron microscopy

As shown in Figure 3.4, scanning electron micrographs of networks, known to be of different mass-length ratio, were not visually distinguishable. The micrographs show great depth of field, so it was not possible to use morphometric analysis in scanning electron micrographs to quantify fibre thickness.

3.4 DISCUSSION

The reproducibility of methods is of paramount importance in the selection of techniques suited to this study. The experiments described in this Chapter show that the techniques used in this thesis vary little from day to

day (Table 3.1).

Measurements made using the turbidimetric technique can be analysed in a variety of ways to give useful information. From the ratio of turbidities may be obtained the network opacity ratio (Dhall and Bryce, 1970) which is indicative of fibre diameter. From the turbidity and fibrin concentration the mass-length ratio of network fibres may be calculated (Carr and Hermans, 1978). In addition, continuous measurements of turbidity may be used to determine lag time (Hantgan and Hermans, 1979). The technique is simple and highly reproducible.

The μ_T may be calculated from the turbidity at 608 nm at physiological fibrin concentrations (Appendix 2). At higher than physiological fibrin concentrations, turbidity at 608 nm may not be directly proportional to λ^{-3}. Since the theoretical basis of this method no longer holds, calculations of μ_T in the presence of higher fibrin concentrations should be made with caution.

Compaction is a simple technique. However, the coefficient of variation of this method is relatively high (Table 3.1). This arises from the low volume of fluid expelled from purified fibrin clots (often 0.05 - 0.10 ml/ml clot), the difficulty of collecting all the expelled fluid and the accuracy limit on the syringe used to collect and measure fluid. As shown in later chapters, lower coefficients of variation were obtained in clots which

compacted more readily.

The perfusion method allows an index of network permeability, the Darcy constant, to be calculated (Appendix 3). The Darcy constant and the network protein concentration are then used to derive the mass-length ratio of the network fibres assuming a value of 10 for the fibre packing constant of the network (Carr et al., 1977). Measurement of permeability is technically demanding. It was feared that the perfusion of incompletely developed networks could lead to changes in flow rate due to unclotted fibrinogen affecting the viscosity of the perfusion fluid. However, significant changes in flow rate with perfusion volume were not observed (Figure 3.2). This is also supported by Carr et al. (1977). Thus, μ_p can be easily calculated from measurements of network permeability.

Scanning and transmission electron microscopy have been previously used to visualize the fibrin network (Carlin et al., 1976; Krause and Zimmerman, 1979; Tesch et al., 1979). However, these techniques are unsuited for the routine examination of fibrin network structure. As Carlin et al. (1976) also found, scanning electron photomicrographs of networks made with different ionic strength or pH are visually indistinguishable (Figure 3.4). Routine use of scanning electron microscopy in early studies in the following chapters reinforced this conclusion. Consequently, this technique was abandoned.

Transmission electron microscopy, on the other hand, may be used to measure the individual thickness of fibres in a network (Krause and Zimmerman, 1979; Tesch et al., 1979; Chapter 4). But, a high thrombin concentration is needed to form fibrin networks successfully on grids. This thrombin concentration is incompatible with the experimental design (Chapter 2) in which the thrombin concentration used was similar to that found at the clotting moment of whole blood, namely, 0.15 U/ml (Shuman and Majerus, 1976). Mainly for this reason, transmission electron microscopy was not used for the routine examination of fibrin networks.

Transmission electron micrographs show that the diameter of network fibres is not homogeneous (Figure 3.3; Krause and Zimmerman, 1979; Tesch et al., 1979). However, in turbidity and permeability methods a single value purporting to be a measure of fibre thickness is obtained. This value must be a mean of many different fibre thicknesses within the network. As will be shown later in this thesis (Chapter 4) turbidity and permeability techniques are influenced in different ways by the distribution of fibre diameters within the network. For this reason both the mechanical methods (μ_p and compaction) and the turbidimetric techniques (μ_T and opacity ratio) have been used in this thesis to investigate fibrin network structure.

Since factor $XIII_a$ crosslinking modifies the

mechanical properties of networks (Gerth et al., 1974) it may be expected that crosslinked networks will behave differently from non-crosslinked networks. This might affect measurements in methods which depend upon mechanical properties of networks, namely, permeability and compaction. Since only networks formed with added Ca^{++} are crosslinked (Appendix 4) fibrin networks were routinely formed both with and without Ca^{++} and then investigated.

Although measurements differ significantly between different batches of fibrinogen these differences did not affect the direction or magnitude of the way in which fibrin network responds to changes in clotting conditions (Figure 3.2). It is, therefore, valid to make observations on networks formed with different batches of fibrinogen. The results and interpretations will remain unaltered.

3.5 CONCLUSIONS

(1) Fibrin network turbidity and permeability may be measured reproducibly. The turbidimetric techniques are more reproducible than the mechanical methods.

(2) Electron microscopy could not be used routinely to study fibrin networks. Networks with known differences cannot be visually distinguished using scanning electron microscopy.

(3) Transmission electron microscopy shows that fibrin network fibre thickness is not homogeneous.

(4) Because of heterogeneous fibre size in fibrin networks and the assumptions and technical limitations of individual methods, a variety of methods should be used to investigate network structure which should be examined in the presence and absence of Ca^{++}.

Table 3.1 The day to day variation and the coefficient of variation of various techniques

Method	Day 1	Day 2	Pooled Data
TURBIDITY			
Lag time (sec)	114±8 (7.0)	126±8 (6.3)	120±12 (10.0)
ΔOD/sec ($x10^{-4}$)	29.1±1.9 (6.5)	31.7±1.7 (5.4)	30.4±2.6 (8.6)
Opacity ratio	0.419±0.006 (1.4)	0.434±0.009 (2.1)	0.426±0.011 (2.6)
$\mu_T \times 10^{12}$ (daltons/cm)	12.0±0.2 (1.7)	12.5±0.4 (3.2)	12.3±0.4 (3.3)
PERMEABILITY			
Fibrin conc. (mg/ml)	2.99±0.02 (0.7)	2.98±0.02 (0.7)	2.98±0.02 (0.7)
$\mu_p \times 10^{12}$ (daltons/cm)	13.0±0.9 (6.9)	15.4±1.2 (7.8)	14.2±1.6 (11.3)
COMPACTION			
Expelled volume (%)	5.2±0.8 (15.4)	4.5±0.5 (11.1)	4.8±0.7 (14.6)

Results show the mean of 3 observations ± SD (coefficient of variation) for two experiments performed on consecutive days and for the pooled data for both days. Networks were made without additional divalent cations. Except for compaction, the coefficients of variation are typical of those obtained throughout this thesis. Lower coefficients of variation were obtained for compaction in later studies.

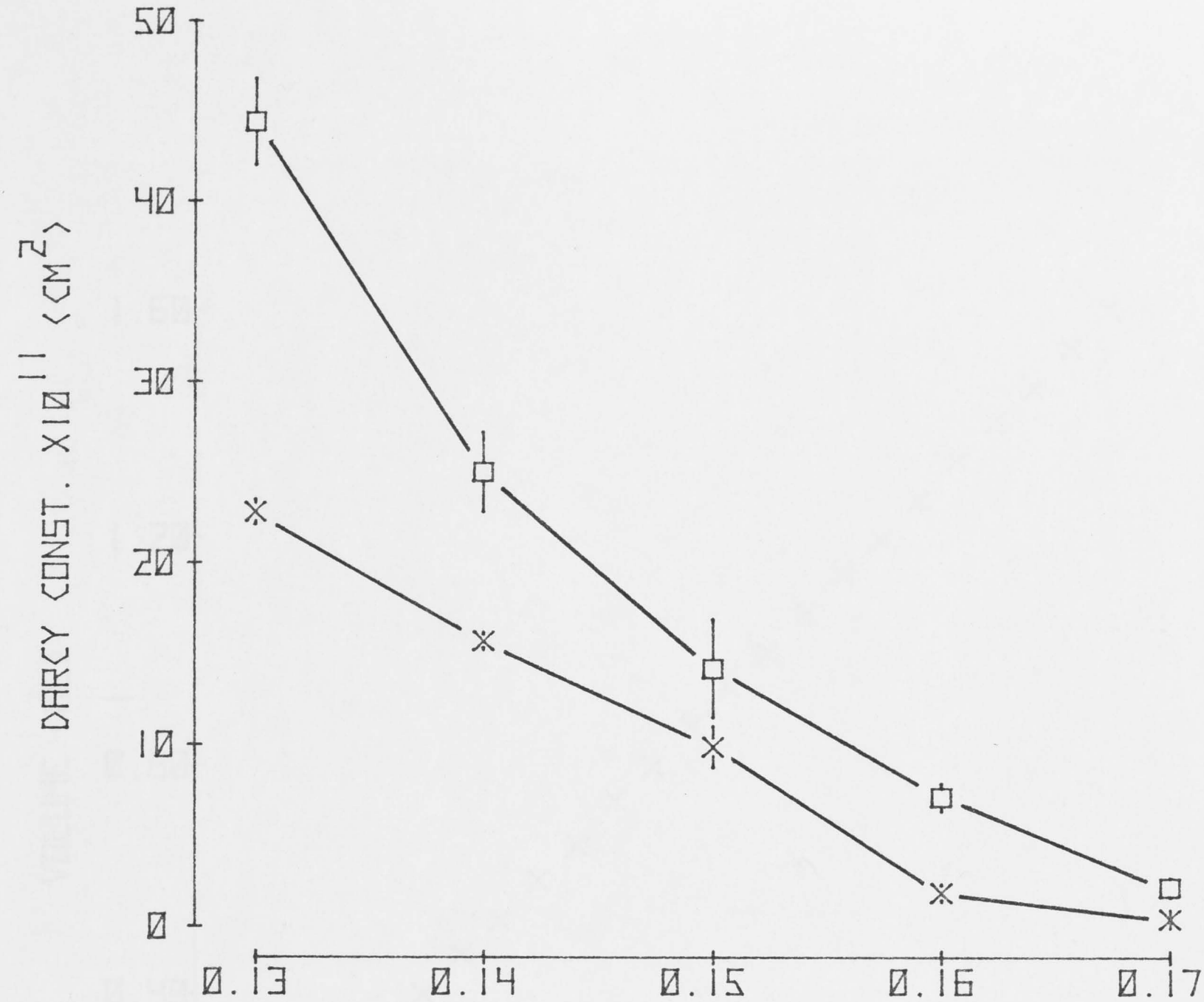


Figure 3.1 The change in Darcy constant with ionic strength in networks made with different batches of fibrinogen and thrombin.

Networks were formed in two different (× and □) fibrinogen solutions which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ of varying ionic strength (Chapter 6). Similar results were obtained where networks did not contain divalent cations or where networks contained 2.02 mM $MgCl_2$. The Darcy constants of networks formed from these batches of fibrinogen were linearly correlated ($r = 0.987$, $p(F) < 0.001$, $n = 15$). Results are the mean of 5 determination $\pm$ SD.

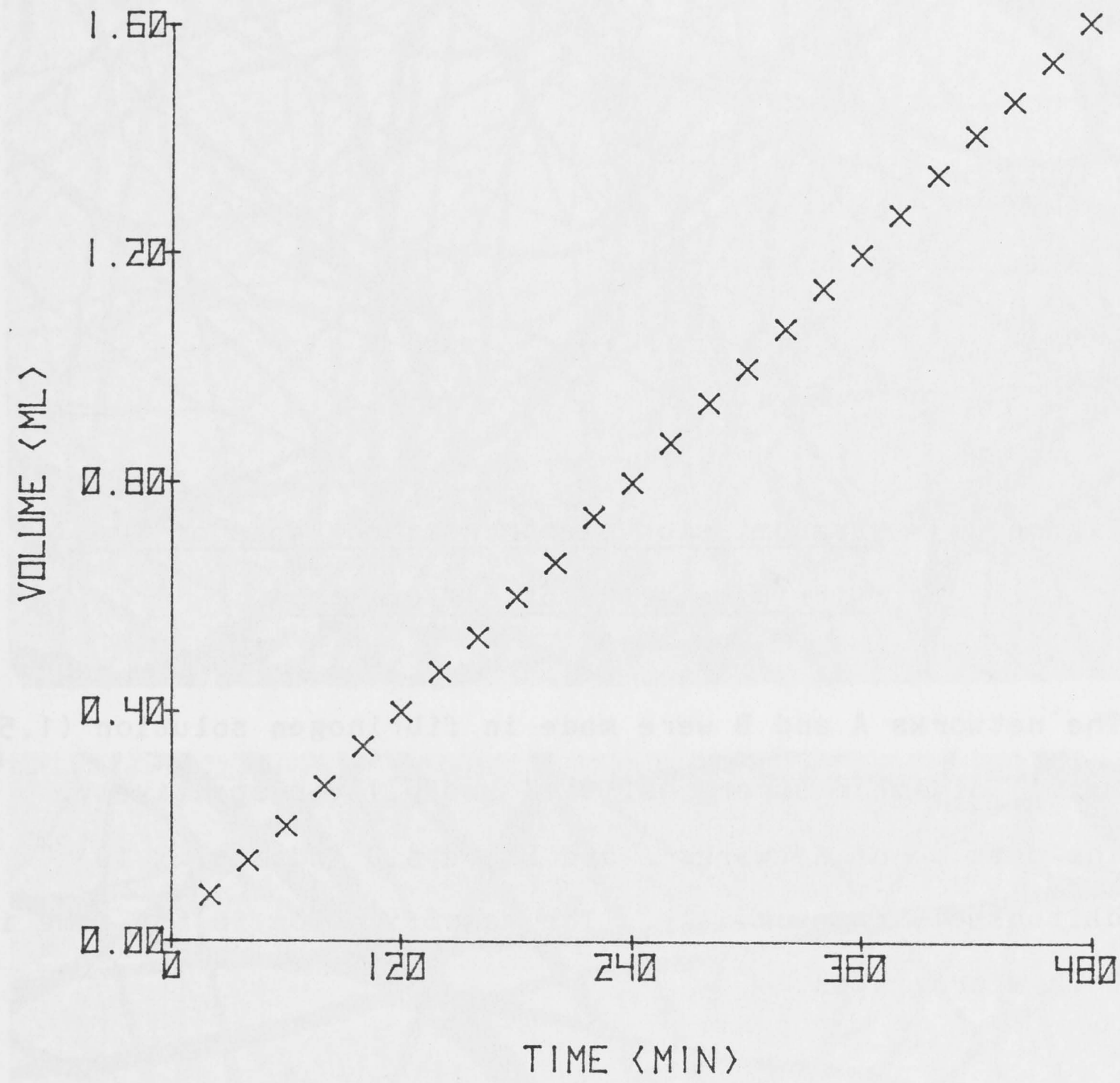


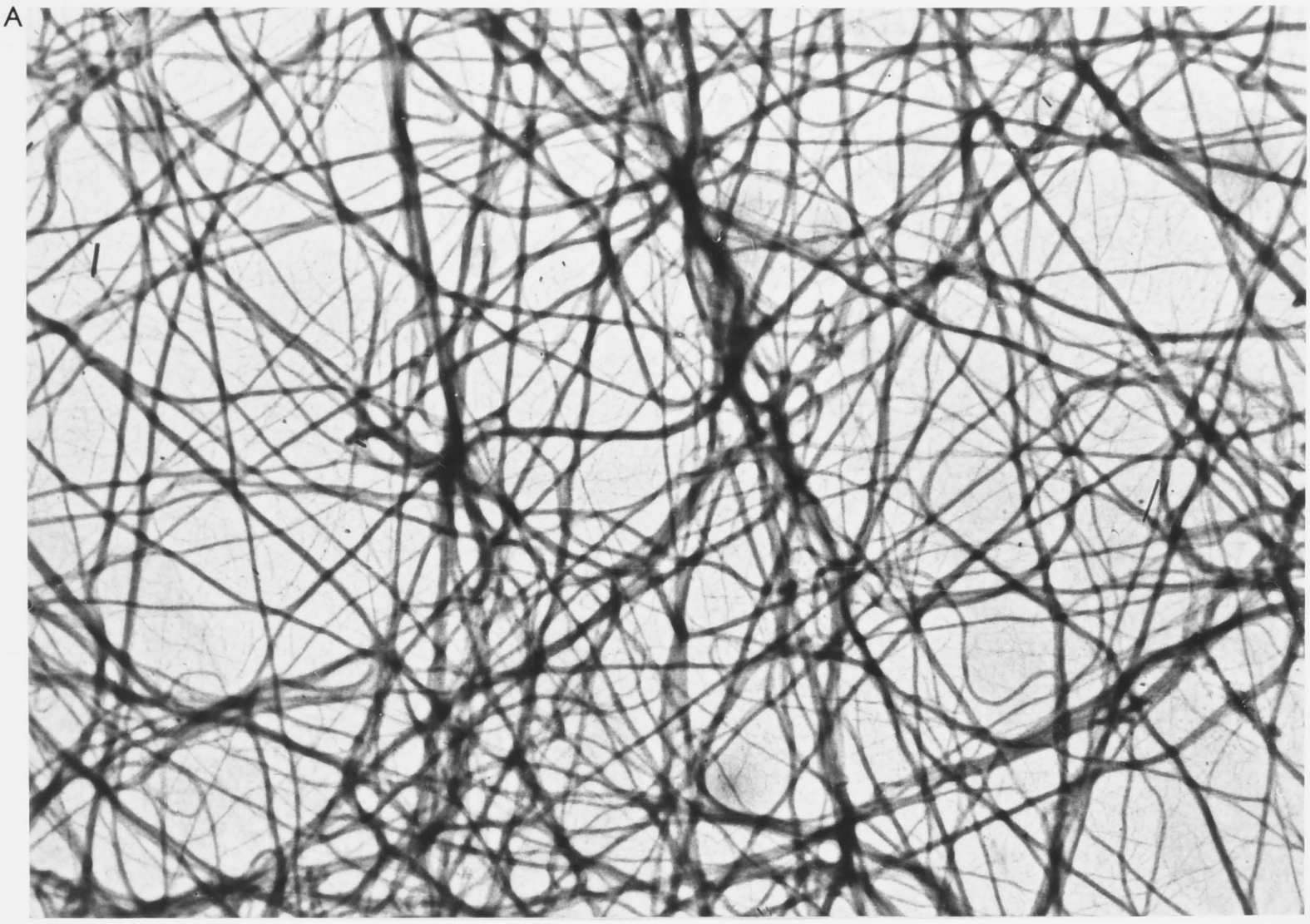
Figure 3.2 A typical time rate change in the volume of fluid perfused through a fibrin network

The network was formed in fibrinogen solution (3.3 mg/ml; pH 7.35; ionic strength 0.133) using 0.15 U/ml thrombin and perfused at 30 minutes. The data are typical of those obtained throughout perfusion studies in fibrin networks.

Figure 3.3 Transmission electron micrographs of two fibrin networks of different μ_T

The networks A and B were made in fibrinogen solution (1.5 mg/ml) of ionic strengths, 0.17 and 0.13, respectively. The mean μ_T of networks A and B are 8.0 and 20.7 x 10^{12} daltons/cm, respectively. The magnification is the same in both micrographs.

A



B

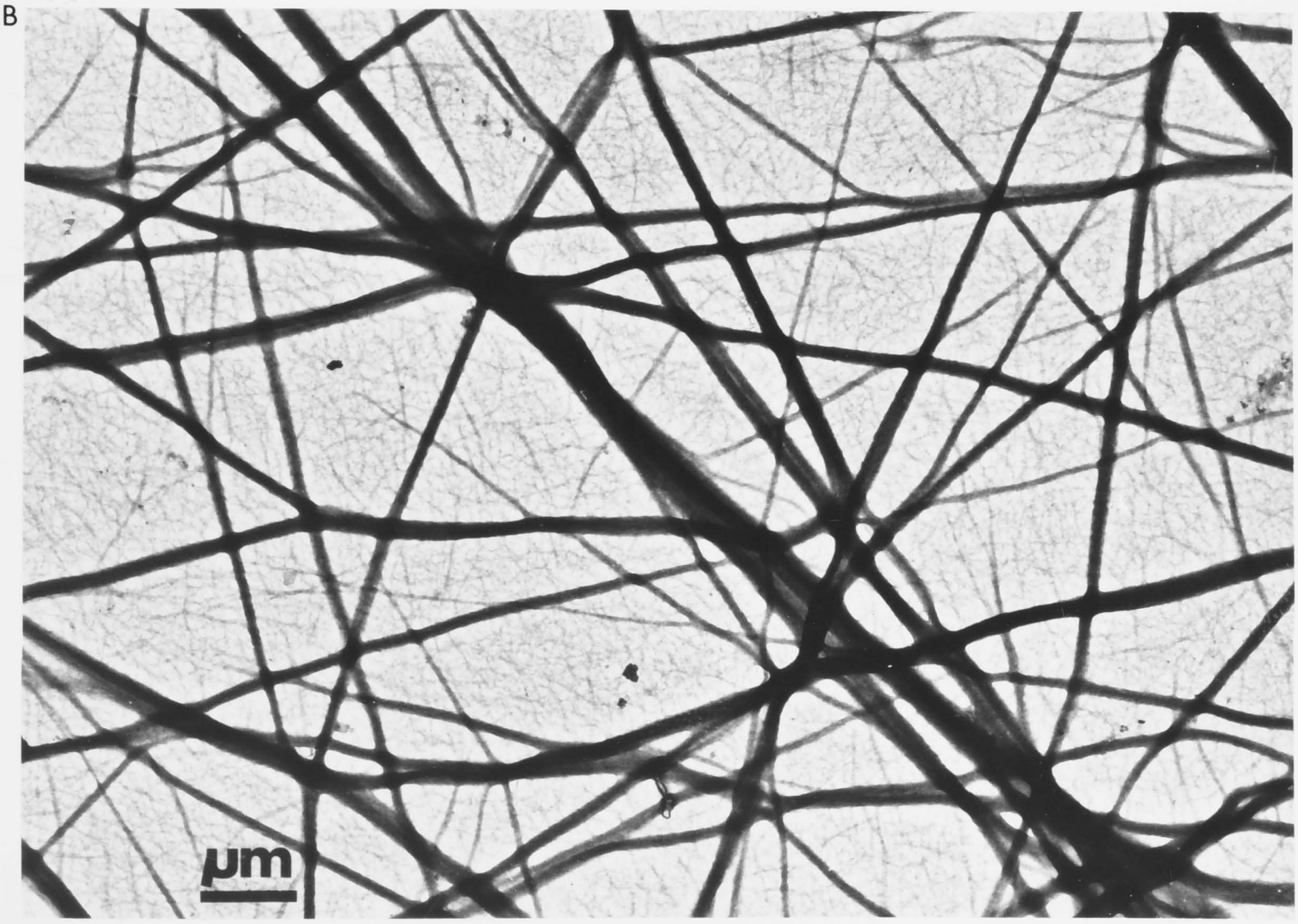
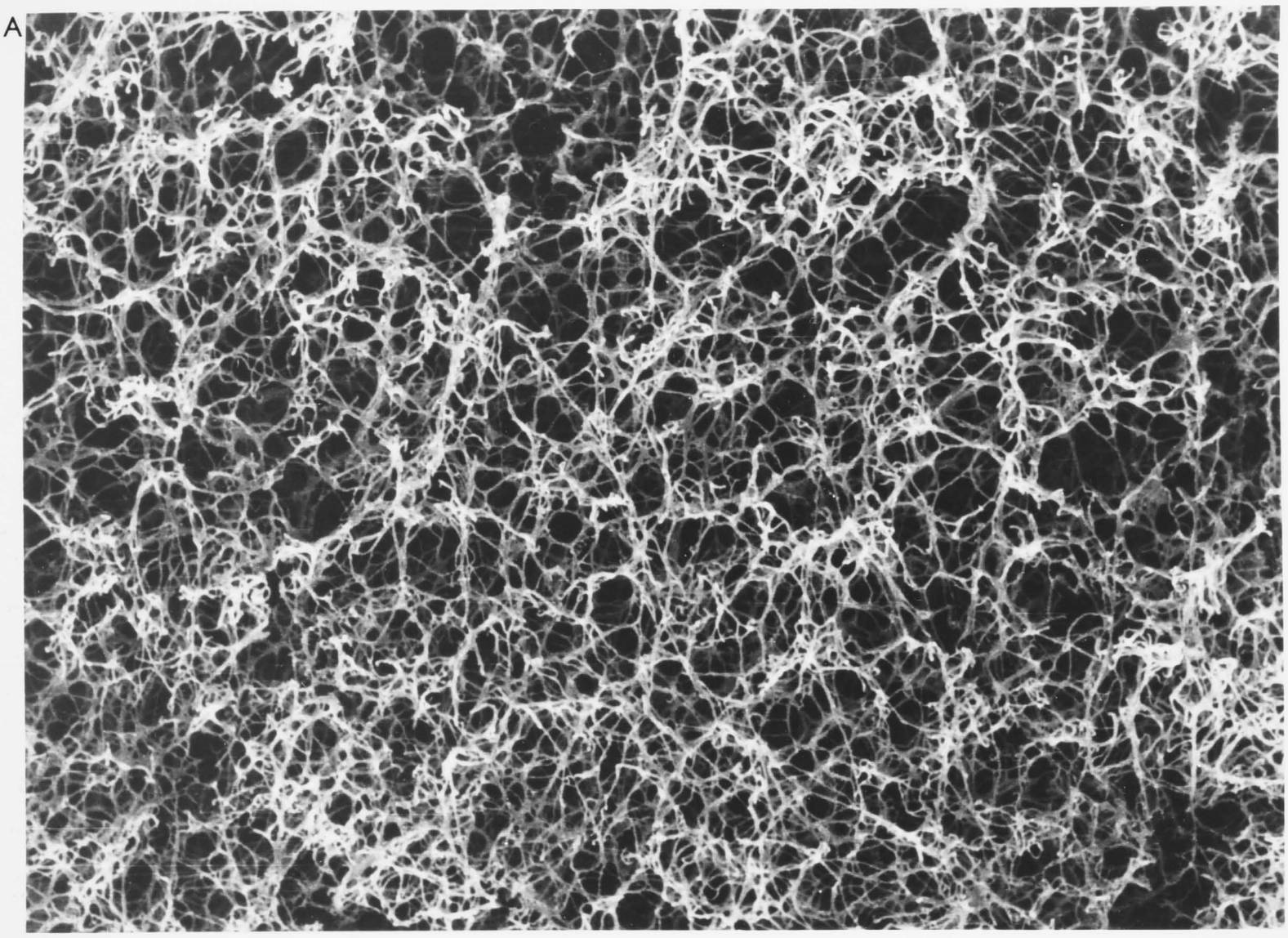


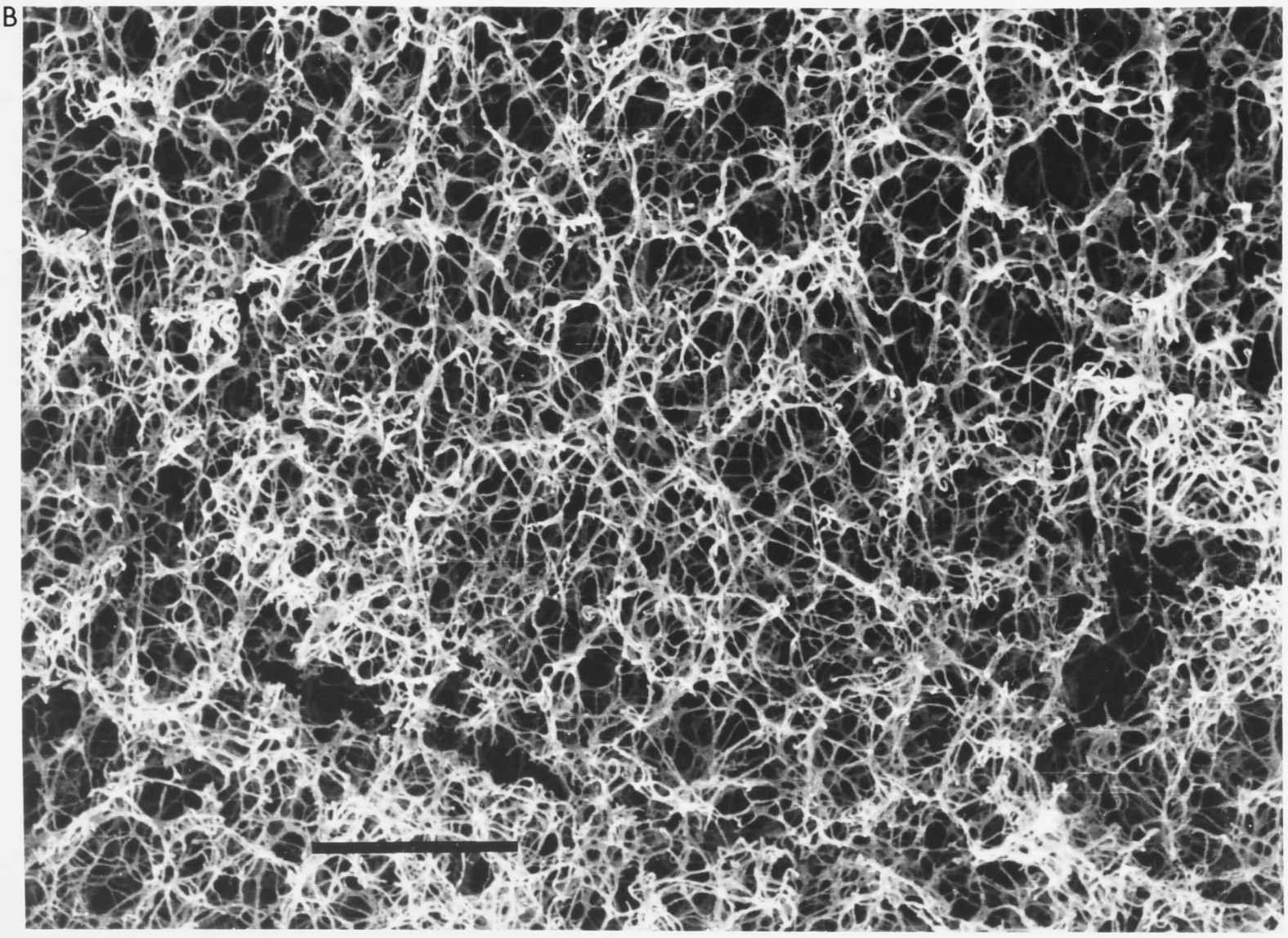
Figure 3.4 Scanning electron micrographs of two fibrin networks of different μ_T.

The networks A and B were made in fibrinogen solution (3.3 mg/ml) of ionic strengths, 0.17 and 0.13, respectively. The mean μ_T of networks A and B are 8.0 and 20.7 x 10^{12} daltons/cm, respectively. The scale is 20 μm and the magnification is the same in both micrographs.

A



B



CHAPTER 4

THE EFFECTS OF FACTOR $XIII_a$ MEDIATED CROSSLINKING ON NETWORK STRUCTURE AND MEASUREMENT OF MASS-LENGTH RATIO

4.1 INTRODUCTION

The mean mass-length ratio of fibres in fibrin networks can be calculated from measurements of either the permeability (Carr et al., 1977) or the turbidity (Carr and Hermans, 1978). Since the mass-length ratio is proportional to the square of the fibre radius, the mean fibre diameter can be calculated provided the fibre density is known. However, the theoretical background rests on the assumption that the diameter of the fibres in a clot is uniform. But this asssmption is not supported by evidence from electron micrographs (Chapter 3). In this Chapter the consequences of polydispersion in the diameter of the fibres on the mass-length ratios calculated from the permeability and the turbidity of fibrin clots are investigated.

4.2 MATERIALS AND METHODS

Fibrin clots were formed from fibrinogen solutions (3.3 mg/ml) which did not contain divalent cations or which contained 2.02 mM $MgCl_2$ or 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$, as described fully in Chapter 2. The pH of the fibrinogen solutions was varied as described in Chapter 6. The mass-length ratio was determined from permeation and turbidity.

Transmission electron micrographs were obtained using the negative staining technique (Raju, 1978). Freshly glow-discharged grids were touched onto the surface of

perfused and unperfused clots (from permeation studies). After 15 seconds, several drops of aqueous uranyl acetate were applied to the grid which was subsequently dried. Grids were examined as described in Chapter 2.

The distribution of fibre diameters was determined using transmission electron micrographs at a final magnification of 70,000X prepared as described in Chapter 2. Grid lines 2 cm apart were drawn and the diameters of all fibres intersecting the lines measured.

4.3 RESULTS

Figure 4.1 shows a plot of μ_p against μ_T. Although μ_p was higher than μ_T, a good linear correlation was obtained ($r = 0.994$) with networks made in the absence of calcium ($n = 10$). However, in networks made in the presence of calcium μ_p was lower and the values do not lie on this regression line. When data from the latter networks was included in a composite regression analysis the linear correlation deteriorated ($n = 15$, $r = 0.633$) and this deterioration was statistically significant (Snedecor and Cochran, 1978). Similar results were also obtained when the networks were formed with a tenfold increase in thrombin concentration (i.e., 1.5 U/ml) and where clotting conditions other than pH are varied.

Figure 4.2 shows the distribution of fibre diameters in a typical fine fibrin network as determined by morphometric

analysis of transmission electron microscopy. The diameters are polydisperse and their distribution is bimodal. On the basis of their diameters the fibrin strands appear to form two relatively distinct networks which have been termed major and minor networks.

Figure 4.3 shows electron micrographs of fibrin clots before and after perfusion. The minor network can be seen in unperfused clots made in the presence of magnesium ions (B) or calcium and magnesium ions (A). However, following perfusion, the minor network is no longer seen in the clot made in the presence of magnesium ions (D). Clearly, the minor network is washed away during permeation studies in the clot made in the presence of magnesium ions. In contrast, the minor network remains relatively intact after perfusion in the clot made in the presence of calcium and magnesium ions (C). Note that only clots formed in the presence of added Ca^{++} are cross-linked by factor $XIII_a$ (Appendix 4).

4.4 DISCUSSION

Morphometric analysis of electron micrographs show that the diameters of the fibrin strands in a clot are polydisperse and that the distribution of fibre diameters is bimodal (Figure 4.2). Thus, the fibrin strands may be separated into two networks according to their fibre diameter and these have been called the major and minor networks. The fibrin strands in the minor network which

correspond to the first peak in the distribution are thinner than those in the major network. This finding is contrary to the assumption implicit in the methods which have been developed to determine the mass-length ratio of fibres in fibrin networks.

The consequences of polydispersity in the diameters of fibrin strands on μ_p and μ_T were investigated (Appendix 5). As a first approximation, a simplified model has been used which considers two superimposed monodisperse networks with diameters of fibrin strands which differ by a factor of ten. This model has shown that the turbidity of the clot will be determined by the major network whilst its permeability or Darcy constant will be markedly reduced by the strands in the minor network. Consequently, corresponding to a given μ_T, μ_P should be smaller in the presence of the minor network than in its absence.

The mass-length ratios calculated from the permeability of the clots are generally greater than those calculated from their turbidity (Chapters 6 and 7). This is consistent with previous observations (Carr et al., 1977). The reasons for this discrepancy are not completely understood but may be related at least in part to the assumption that the packing constant has a value of 10 (Carr et al., 1977). However, in clots made without calcium ions a good linear correlation was obtained between μ_T and μ_P. In these clots the minor network did not withstand the permeation pressure and was washed away

(Figure 4.3), so that the permeability of the clot would have been determined predominantly by the major network. Since the contribution of the minor network to turbidity is negligible in these clots (Appendix 5) the values of μ_P and μ_T may be taken to correspond to those of the major network only. Thus, in the absence of the minor network a good linear correlation is obtained between μ_T and μ_P.

The fibrin network is strengthened in clots made in the presence of calcium ions, by factor $XIII_a$ mediated crosslinking (McDonagh et al., 1971). This imparts additional strength to the minor network which then seems able to withstand the permeation pressure used in this investigation (Figure 4.3). Thus, in these clots the minor network offers additional resistance to flow, their permeability is lowered and the mass-length ratio calculated from the permeability is reduced. In keeping with this observation, μ_P of clots made in the presence of calcium was smaller than expected from the corresponding mass-length ratio calculated from turbidity (Figure 4.1).

Although the discussion has been restricted to two networks, similar considerations apply in an actual clot which may be thought of as a superposition of a series of monodisperse networks. In general, as the diameter of the strands in a network decreases, its contribution to turbidity will decrease while its resistance to flow will increase. Thus, calculations of mass-length ratio based on measurements of turbidity will give a value greater than

the mean value. On the other hand calculations based on measurements of permeability will give a mean mass-length ratio less than the true mean. The difference between the maximum and minimum fibre diameters in a clot may be greater or less than that assumed in the two network model (Appendix 5) and this will determine the magnitude of the error between the calculated and true mean.

The effect of variation in fibre diameter within the major network alone on measurements of mass-length ratio is not large and may be negligible because estimates of mass-length ratio of the fibres based on the two techniques give good correlation when the minor network is not stabilized by crosslinking.

It may be that the consequences of polydispersion become evident only when the minor network is stabilized. However, in general, due to polydispersion in the diameters of the fibrin strands, an error will be introduced in calculations of mass-length ratio whose magnitude will depend upon the range of diameters and the shape of the distribution. Thus, to overcome pitfalls associated with a single technique, more than one technique should be used to examine and characterize properties of fibrin network.

4.5 CONCLUSIONS

(1) The diameters of the strands in a fibrin network are polydisperse and when measured experimentally show a bimodal distribution.

(2) Turbidity and permeation techniques are sensitive to opposite ends of the distribution of fibre thicknesses; the thinnest fibres which contribute little to the turbidity of a network have a marked influence on its permeability when strengthened by crosslinking.

(3) Crosslinked clots have lower permeability than non-crosslinked clots.

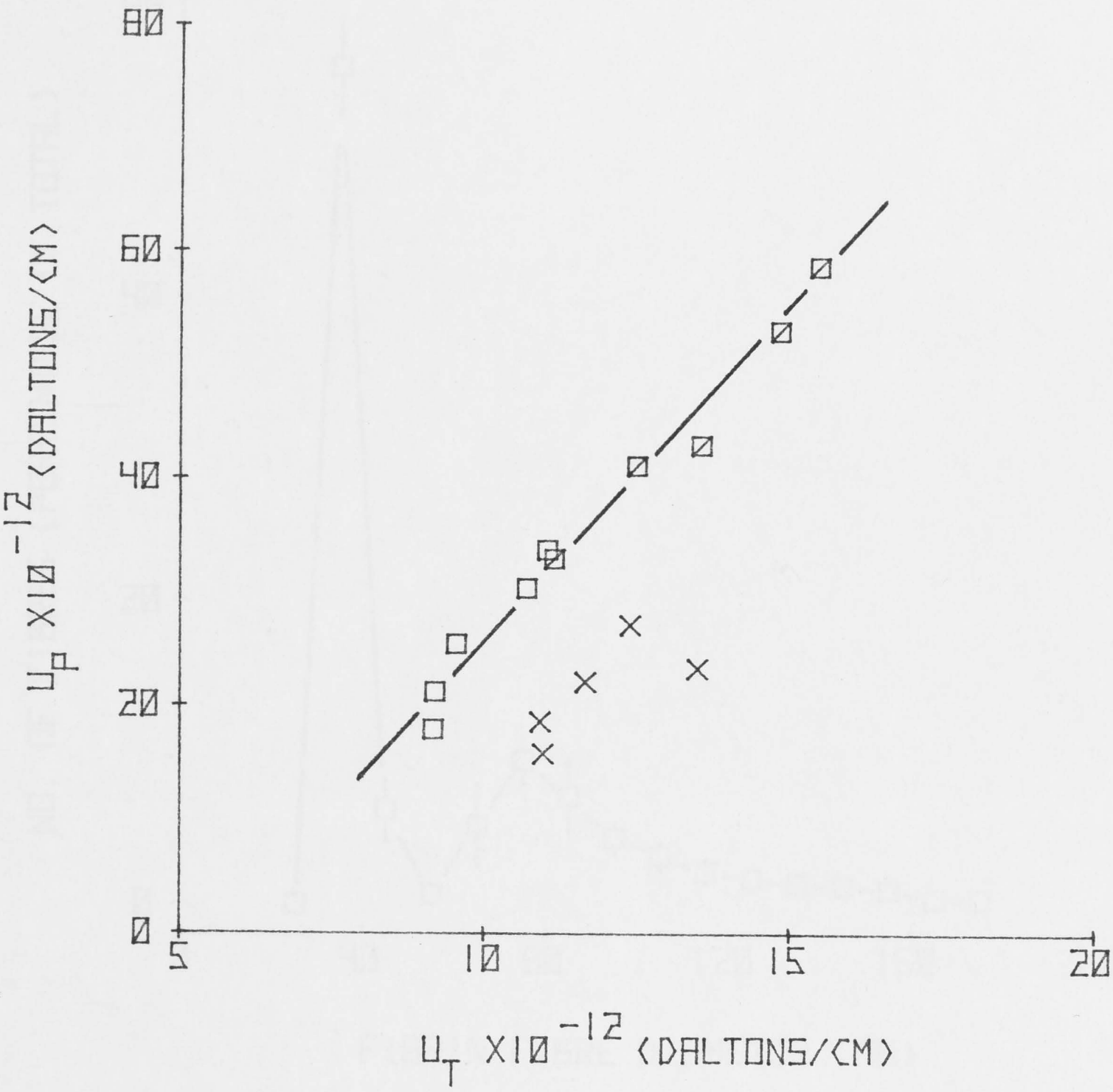


Figure 4.1 Correlation between μ_p and μ_T where pH is varied

Fibrinogen solutions (3.3 mg/ml) were clotted in the presence of magnesium ions (☑), calcium and magnesium ions (X) and without divalent cations (□).

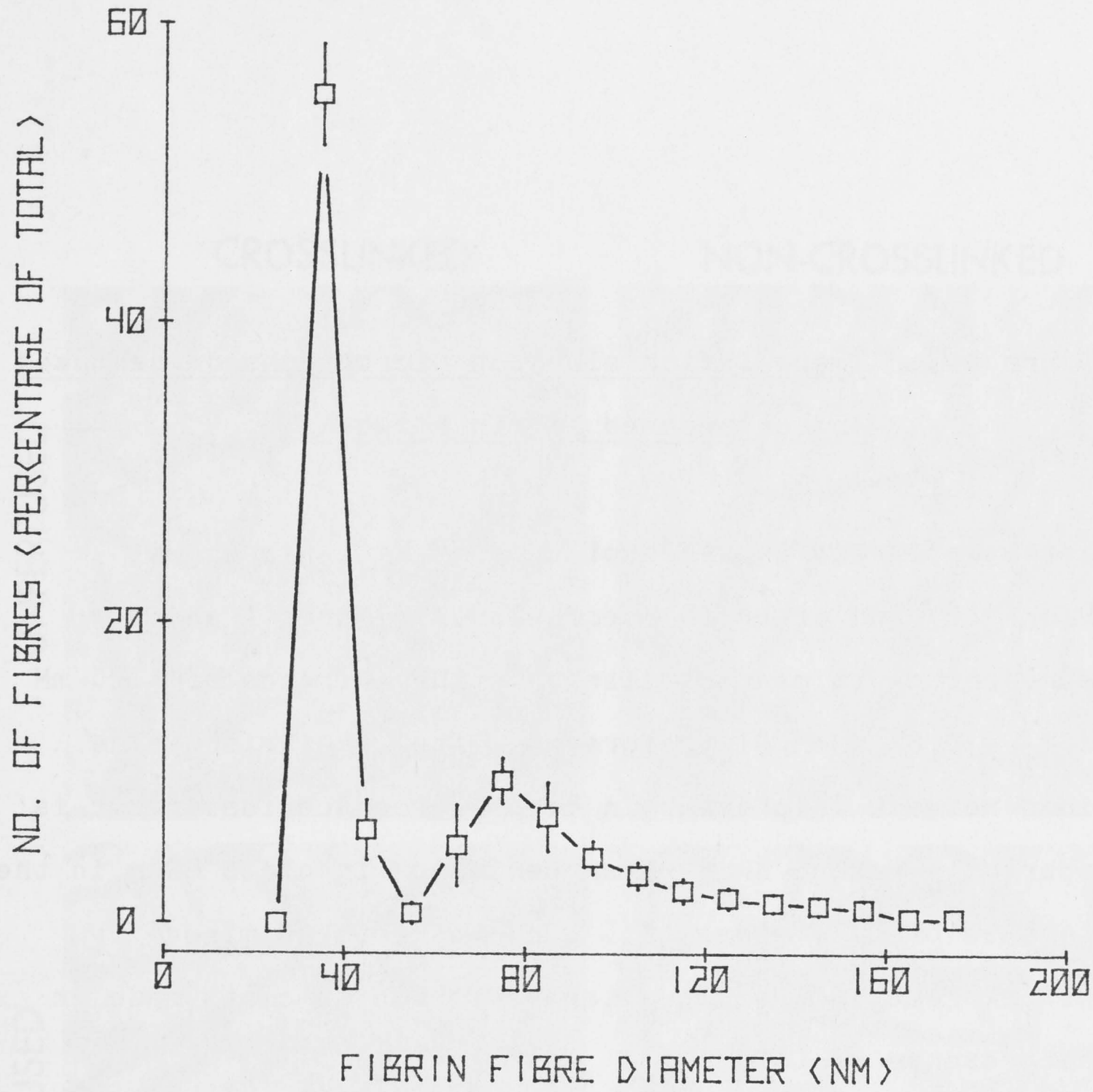


Figure 4.2 Distribution of fibre diameters in a typical fine fibrin network

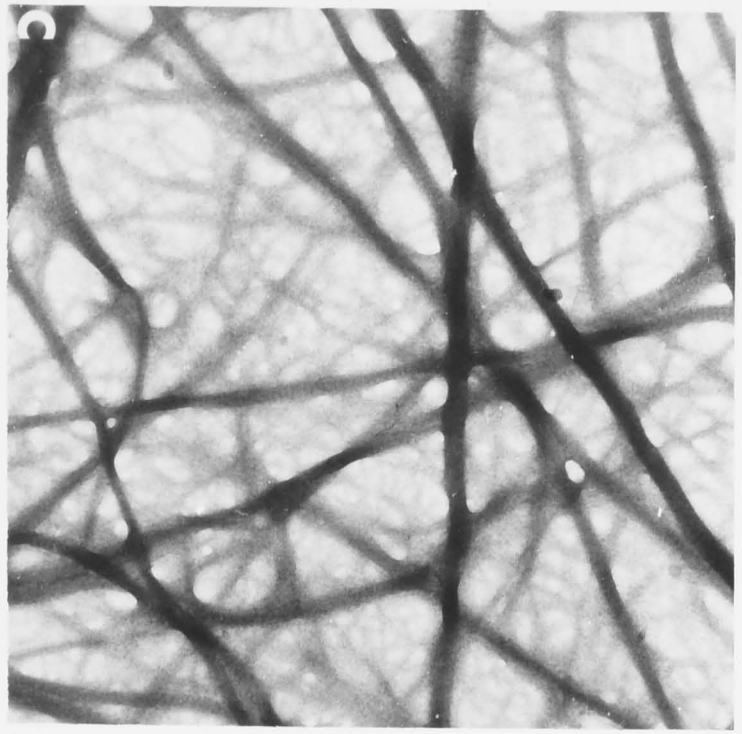
Fibre diameters were determined by morphometric analysis of transmission electron micrographs. Results are the mean of three random sections.

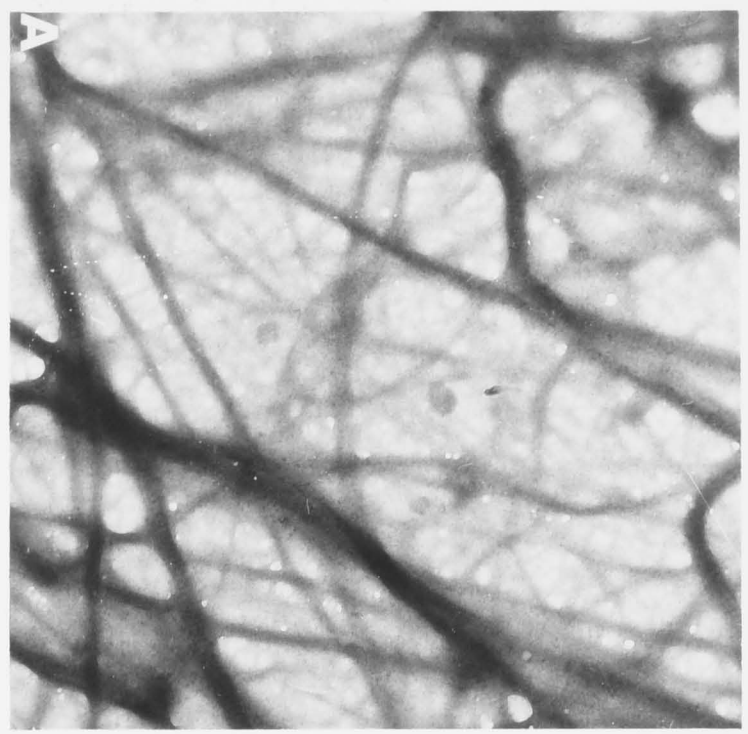
Figure 4.3 Transmission electron micrographs of perfused and unperfused fibrin networks

Clot made in the presence of 2.02 mM $MgCl_2$ is shown before (B) and after (D) perfusion. Figures A and C represent clots made similarly in the presence of 1.20 mM $CaCl_2$ + 0.82 mM $MgCl_2$ before and after perfusion. The minor network is present in clots before perfusion but is apparently washed away after perfusion in clots made in the presence of Mg^{++} alone (D). In contrast the minor network remains visible after perfusion in clots made in the presence of Ca^{++} (C).

PERFUSED

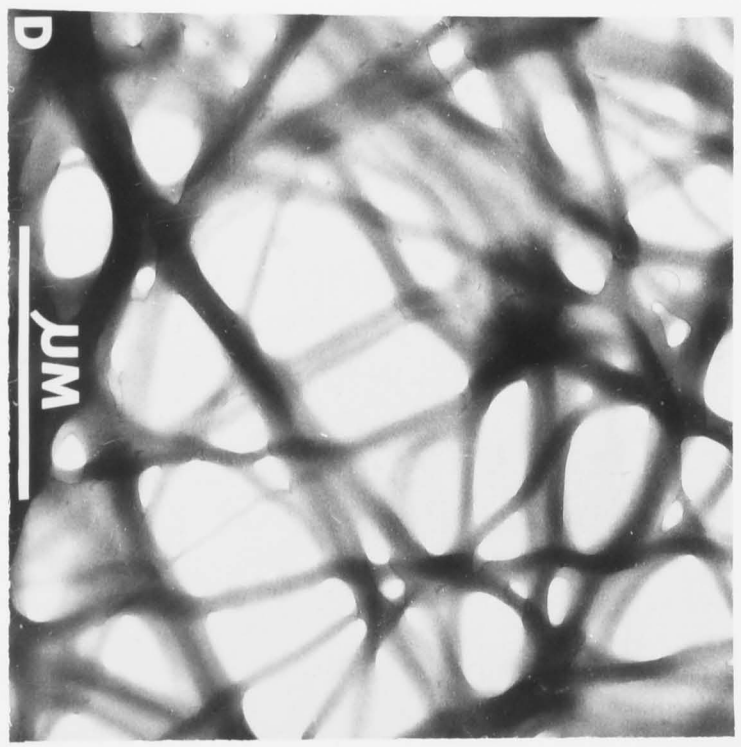
UNPERFUSED

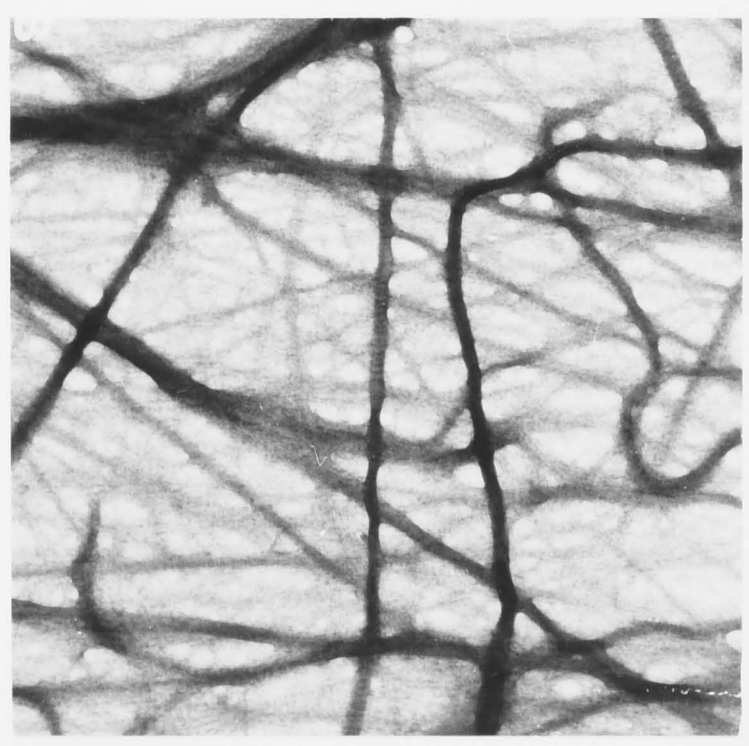




CROSSLINKED

NON-CROSSLINKED





CHAPTER 5

A COMPARISON OF METHODS

5.1 INTRODUCTION

From theoretical reasoning it has been previously argued that opacity ratio (Dhall and Bryce, 1970) and compaction (Dhall et al., 1976) reflect the relative fineness or coarseness of fibres in fibrin networks. However, the theoretical reasoning has not been verified by direct studies. Studies described in this Chapter examine this and some related problems.

5.2 MATERIALS AND METHODS

The data obtained under a variety of clotting conditions with the perfusion, the turbidity and the compaction techniques in Chapters 4 and 5 are compared and critically analysed.

5.3 RESULTS

The changes in lag time, ΔOD/sec, μ_T, μ_p, opacity ratio, permeability, compaction and network protein concentration under a variety of clotting conditions have been described in Chapters 6 and 7. Data from those studies has been used in the following analysis.

5.3.1 Perfusion

The network protein concentration was found to generally decrease slightly but significantly with

permeability (Figure 5.1). The network protein concentration was significantly greater in network made in the presence of Ca^{++}.

5.3.2 Compaction

Compaction generally correlated linearly with mass-length ratio (Figure 5.2). This relationship is influenced by divalent cations.

5.3.3 Turbidity

5.3.3.1 Opacity ratio

The opacity ratio of networks which had developed for 30 minutes was directly proportional to that of more completely developed networks (Figure 5.3).

Opacity ratio generally correlated linearly with μ_T (Figure 5.4).

5.3.3.2 Lag time and ΔOD/sec

Lag time is inversely proportional to ΔOD/sec over a wide range of thrombin concentrations (Figure 5.5).

ΔOD/sec either increased or decreased with μ_T (Figure 5.6). It increased linearly with μ_T when ionic strength was reduced but it decreased with μ_T when thrombin

concentration was reduced.

Lag time likewise increased or decreased with network permeability (Figure 5.7). Lag time was directly related to Darcy constant where thrombin concentration was altered but it was inversely related to Darcy constant where ionic strength was altered.

5.4 DISCUSSION

These studies support and extend the observations made in earlier Chapters. When experimental conditions are manipulated to alter the mass-length ratio the opacity ratio, lag time, ΔOD/sec and compaction correlate with mass-length ratio. However, depending upon the method used these correlations breakdown when the concentration of fibrin or the kinetics of fibrin generation are altered or when networks are crosslinked by factor $XIII_a$. Each method is discussed separately below.

Network protein concentration determined from perfusion studies generally followed network permeability (Figure 5.1). The protein concentration in the perfusate generally depended upon how quickly the network was perfused after initiating the clotting. It is interesting that the fibre thickness in incompletely developed networks mirrors that of more completely developed networks (Figure 5.3). Network protein concentration was significantly increased in the presence of Ca^{++}, presumably due to crosslinked

stable minor network (Chapter 4).

Compaction is greater in coarser than in finer networks (Dhall et al., 1976). Indeed, Figure 5.2 shows that compaction is strongly influenced by mass-length ratio. However, compaction is additionally sensitive to factor $XIII_a$ mediated crosslinking (Chapter 7) and to fibrin concentration (Chapter 6). Thus, compaction is influenced by the tensile properties of the network as well as by the fibre thickness.

The claim that opacity ratio is an index of fibre thickness (Ferry and Morrison, 1947; Dhall and Bryce, 1970) is supported by findings shown in Figure 5.4. However, opacity ratio increases while μ_T decreases when fibrinogen concentration is altered (Chapter 6). Thus, the opacity ratio reflects mass-length ratio only when fibrin concentrations remains unaltered. When fibrin concentration is altered opacity ratio is additionally sensitive to the density of fibrin fibres.

Lag time is determined from and inversely related to ΔOD/sec (Figure 5.5). Except where thrombin concentration is altered, ΔOD/sec increases with μ_T (Figure 5.6). That is, ΔOD/sec and thus lag time, is sensitive to both changes in the rate of polymerization as well as to the extent of lateral aggregation.

Clotting time is the period prior to gelation. It may

vary directly with lag time when the rate of polymerization is varied. However, this may not hold when the extent of lateral aggregation is varied. Blomback and Okado (1982a) determined lag time (termed it clotting time) and found it directly proportional to network permeability under conditions of varying thrombin or fibrinogen concentrations. The data in Figure 5.7 supports, in part, their findings. In addition it shows that lag time is inversely proportional to permeability when ionic strength is varied. The direct relationship between lag time and permeability postulated by Blomback and Okado (1982a), therefore, does not hold under all conditions.

An increase in fibrin concentration increases resistance to permeation and compaction and increases turbidity and hence, opacity ratio, lag time and ΔOD/sec. Unlike turbidimetric methods, the permeation and compaction methods are sensitive to strengthening of the network by factor $XIII_a$ mediated crosslinking. Both mechanical and turbidimetric methods should be used simultaneously when comparing the structures of crosslinked and non-crosslinked networks.

5.5 CONCLUSIONS

(1) Opacity ratio reflects mass-length ratio only when fibrin concentration is not altered.

(2) The direct relationship between lag time and clot permeability postulated by Blomback and Okado (1982a) does not hold under all conditions.

(3) Network compaction is influenced by the tensile properties of the clot as well as by the thickness of fibrin fibres.

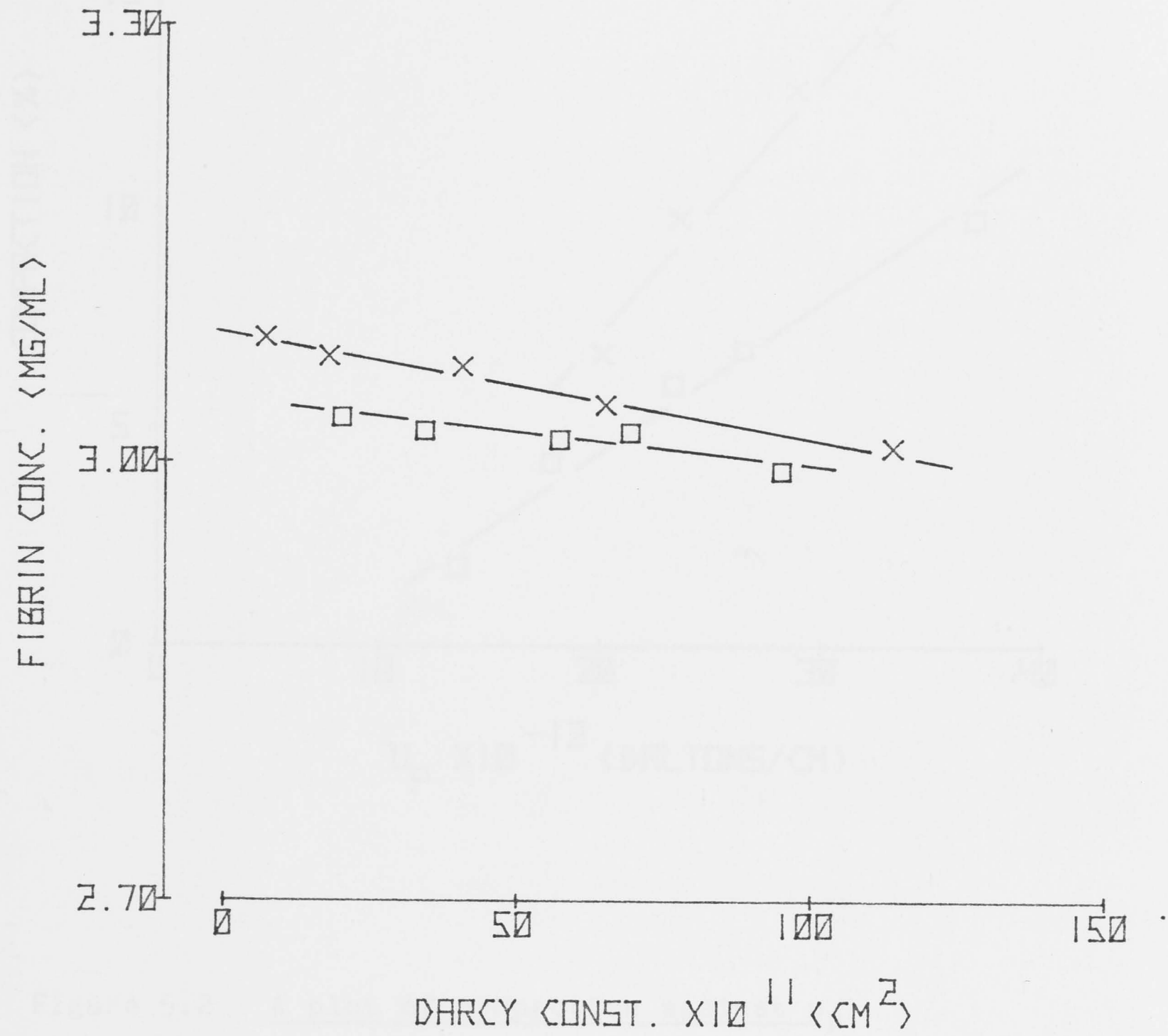


Figure 5.1 A plot of network protein concentration against clot permeability

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Ionic strength was varied (Chapter 6).

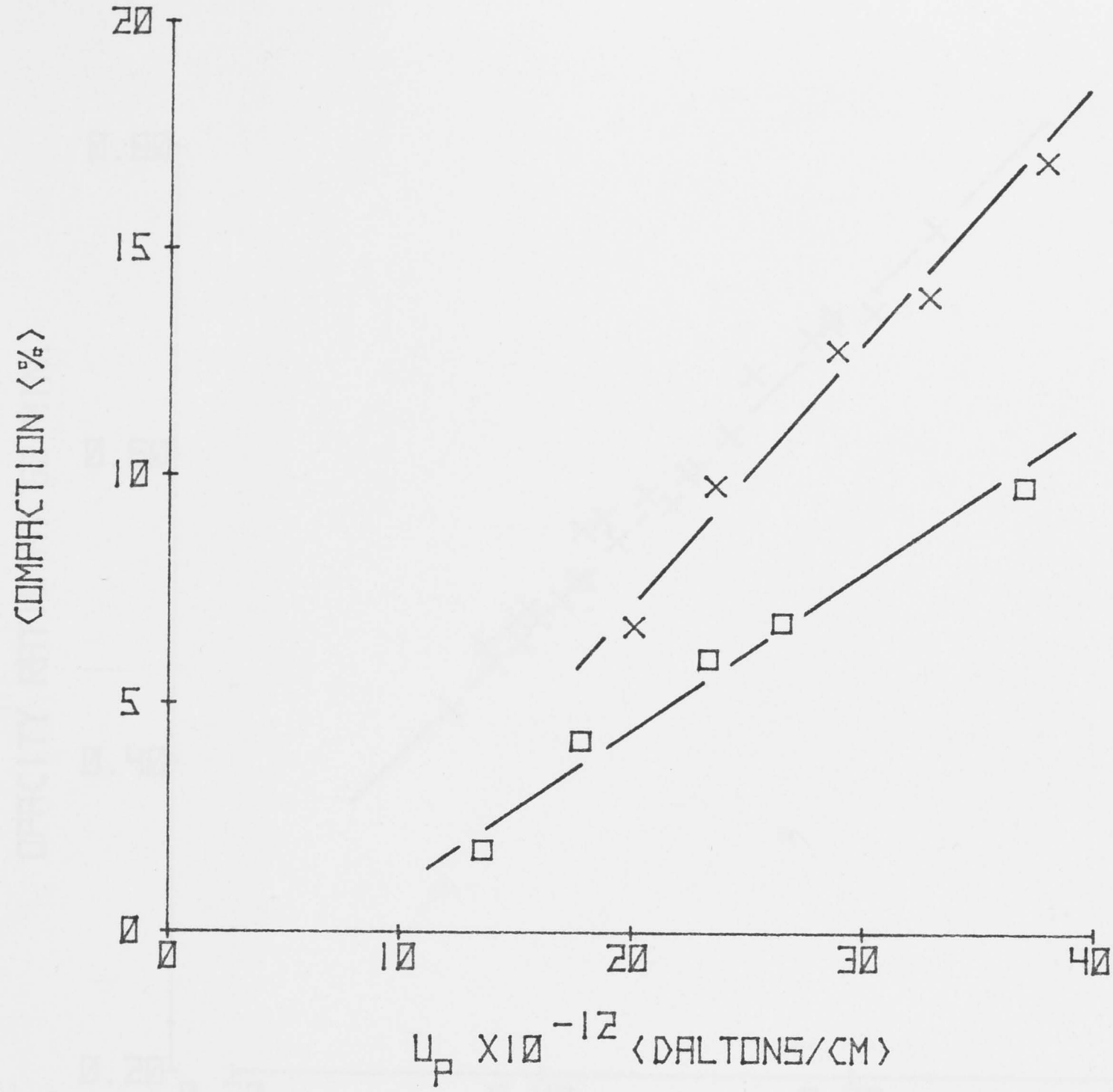


Figure 5.2 A plot of compaction against μ_p

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Thrombin concentration was varied (Chapter 6).

Compaction correlates linearly with u_p (networks made without divalent cations: r= 0.989, p(F) < 0.0015 ; networks made with divalent cations : r = 0.990, p(F) < 0.0012; both sets of data together : r = 0.868, p(F) < 0.0011).

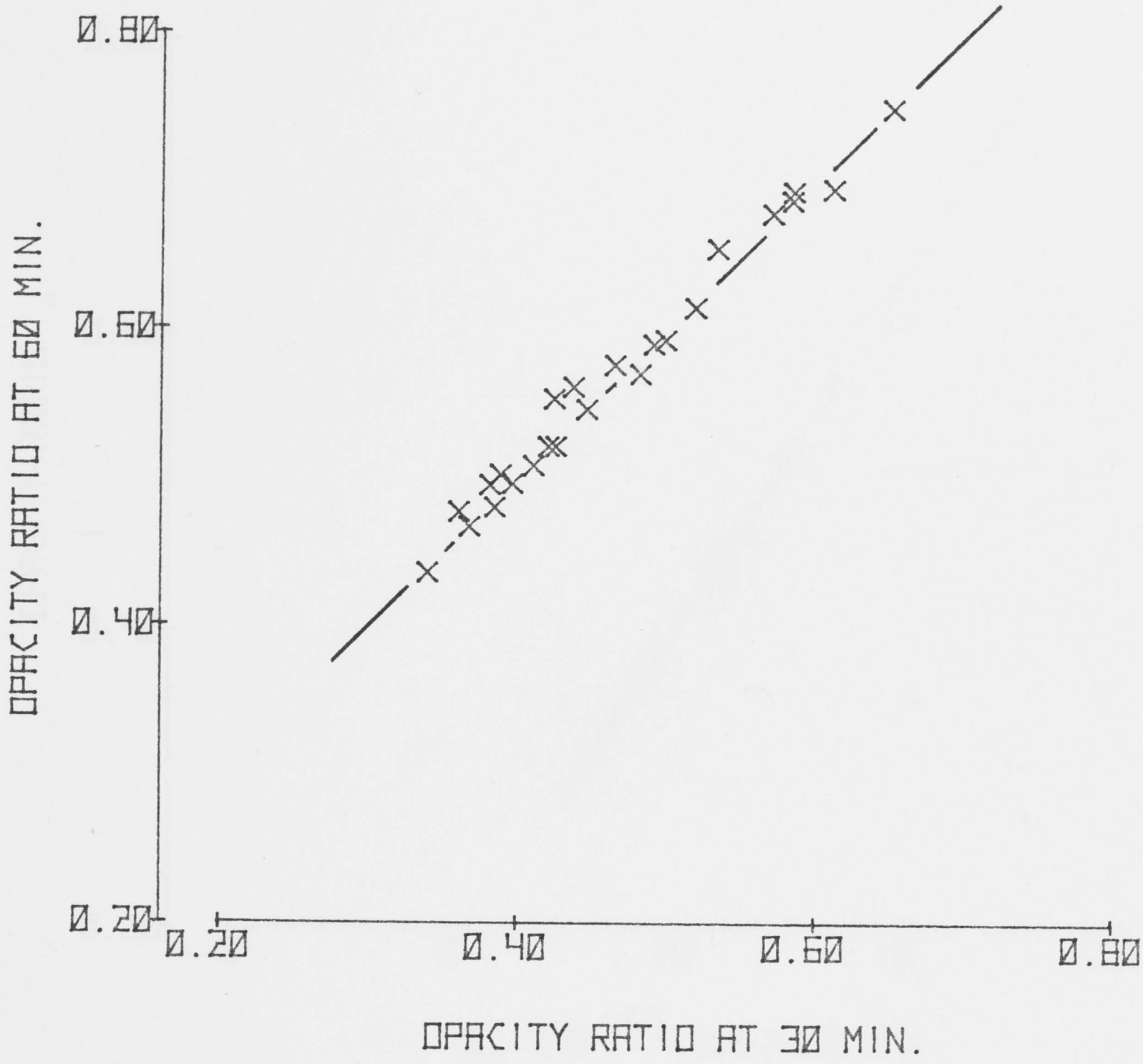


Figure 5.3 The relationship between opacity ratio measured at 30 minutes and at 60 minutes

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations, which contained 2.02 mM $MgCl_2$ or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. Temperature was varied (Chapter 6). Opacity ratio at 30 minutes correlates linearly with that at 60 minutes ($r = 0.990$, $p(F) < 0.0001$, $n = 24$).

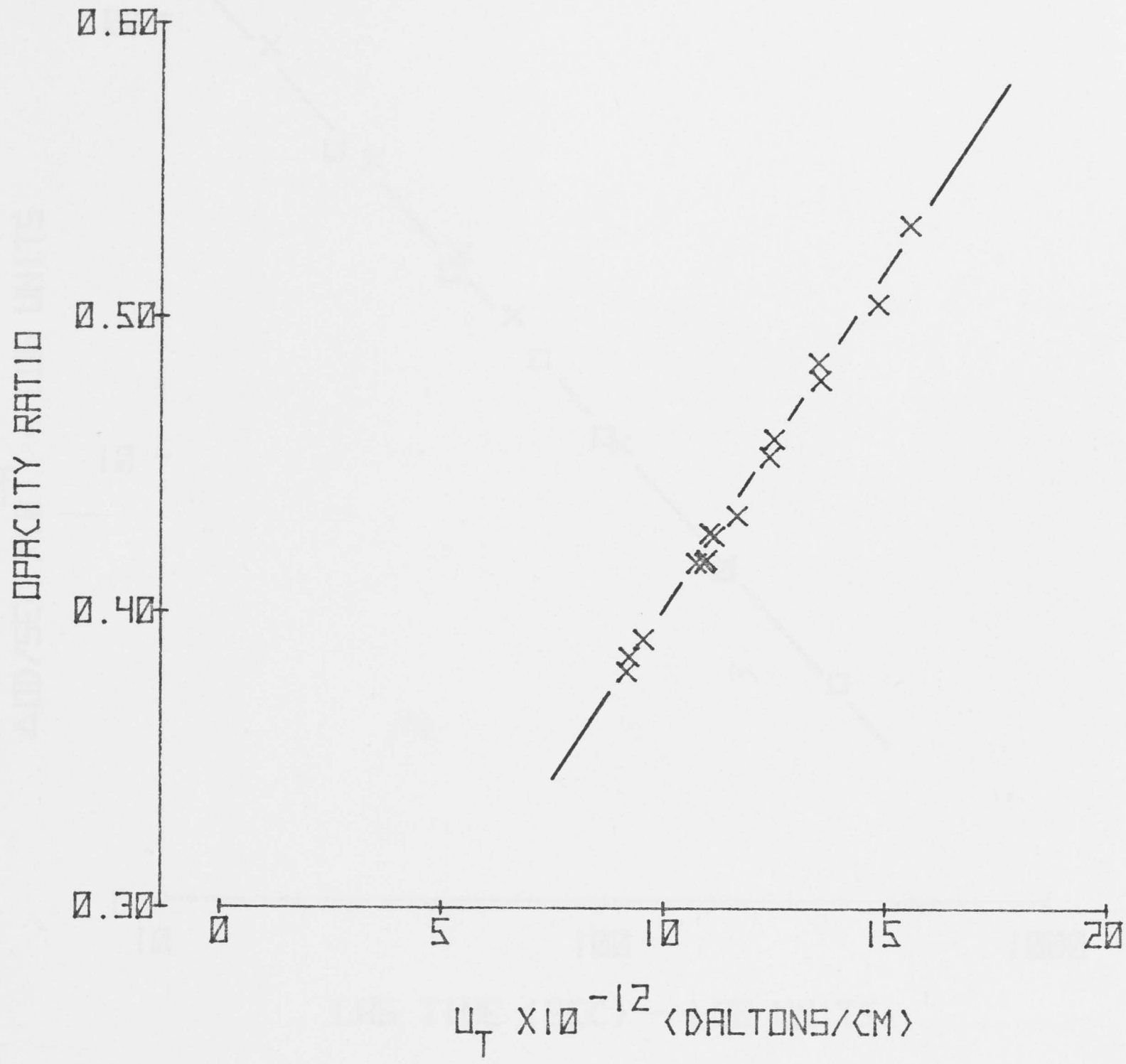


Figure 5.4 A plot of opacity ratio against u_T

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. The pH was varied (Chapter 6).

Opacity ratio correlates linearly with μ_T ($r=0.997$, $p(F) < 0.0001$, $n = 15$).

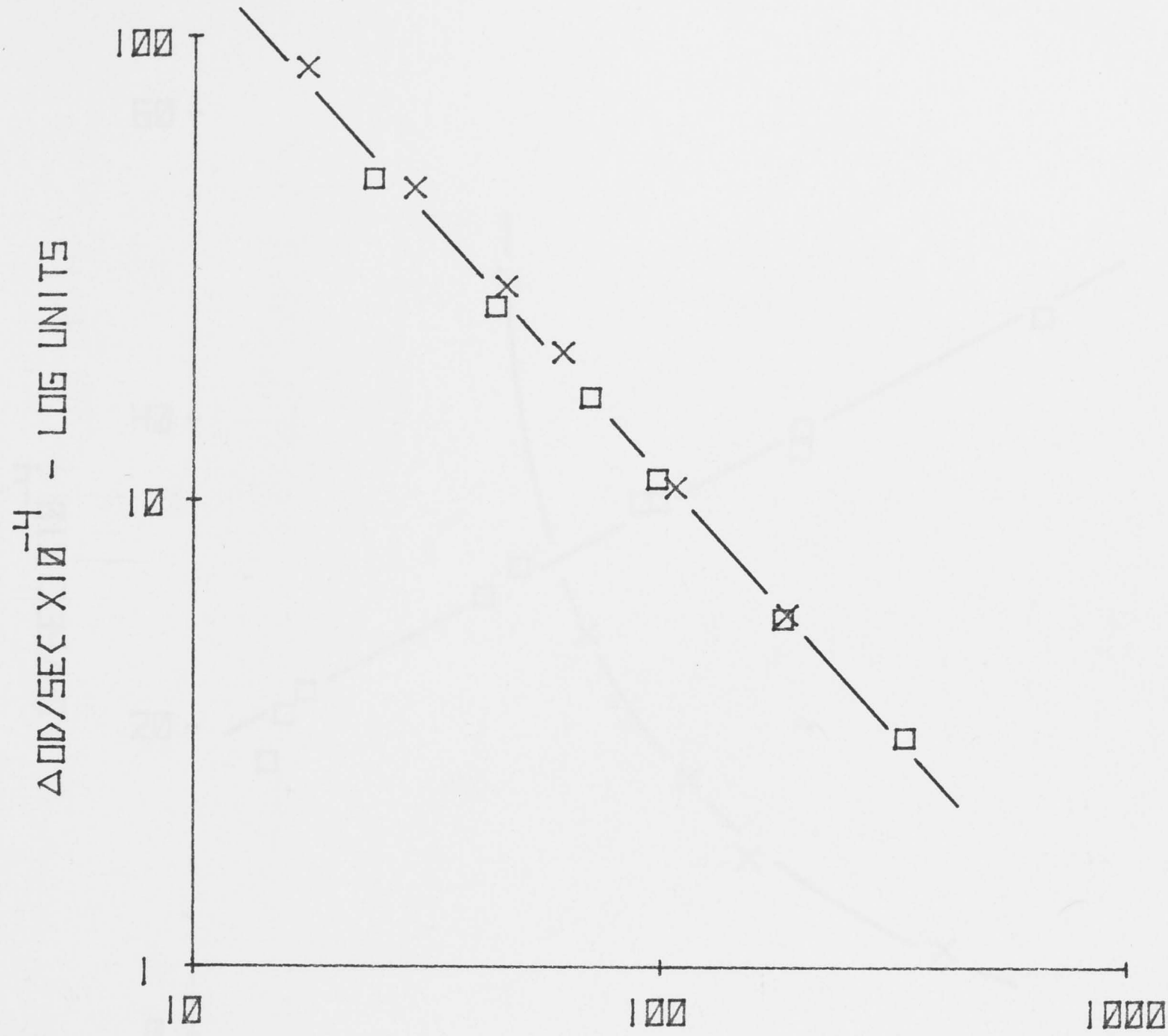


Figure 5.5 A plot of ΔOD/sec against lag time

Networks were formed in fibrinogen solutions (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Thrombin concentration was varied (Chapter 6).

Lag time is inversely related to ΔOD/sec ($r = -0.997$, $p(F) < 0.0001$, $n = 10$).

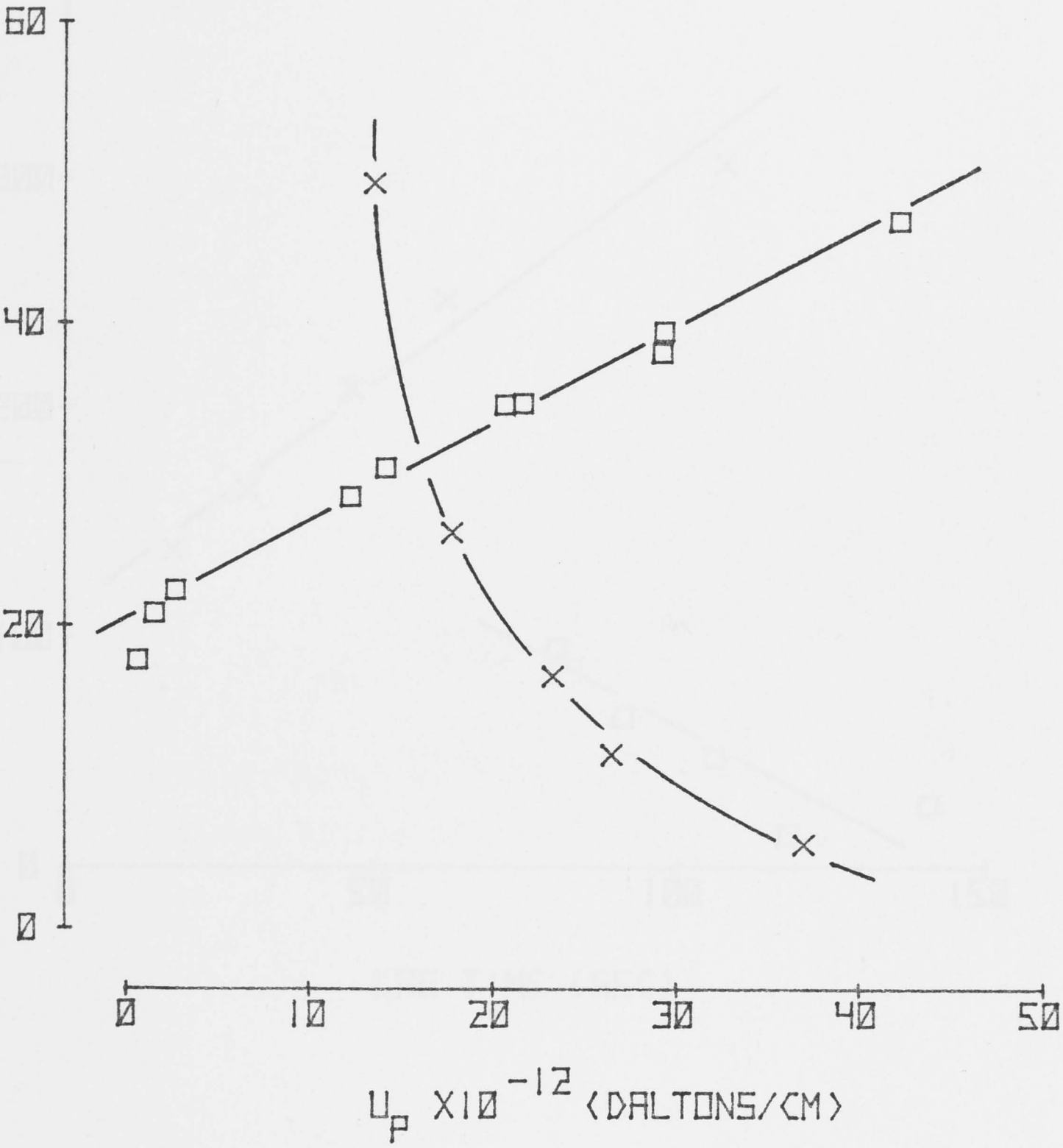


Figure 5.6 A plot of ΔOD/sec against μ_p

Networks were formed in fibrinogen solution (3.3 mg/ml) without divalent cations. Ionic strength (□) or thrombin concentration (×) was varied (Chapter 6).

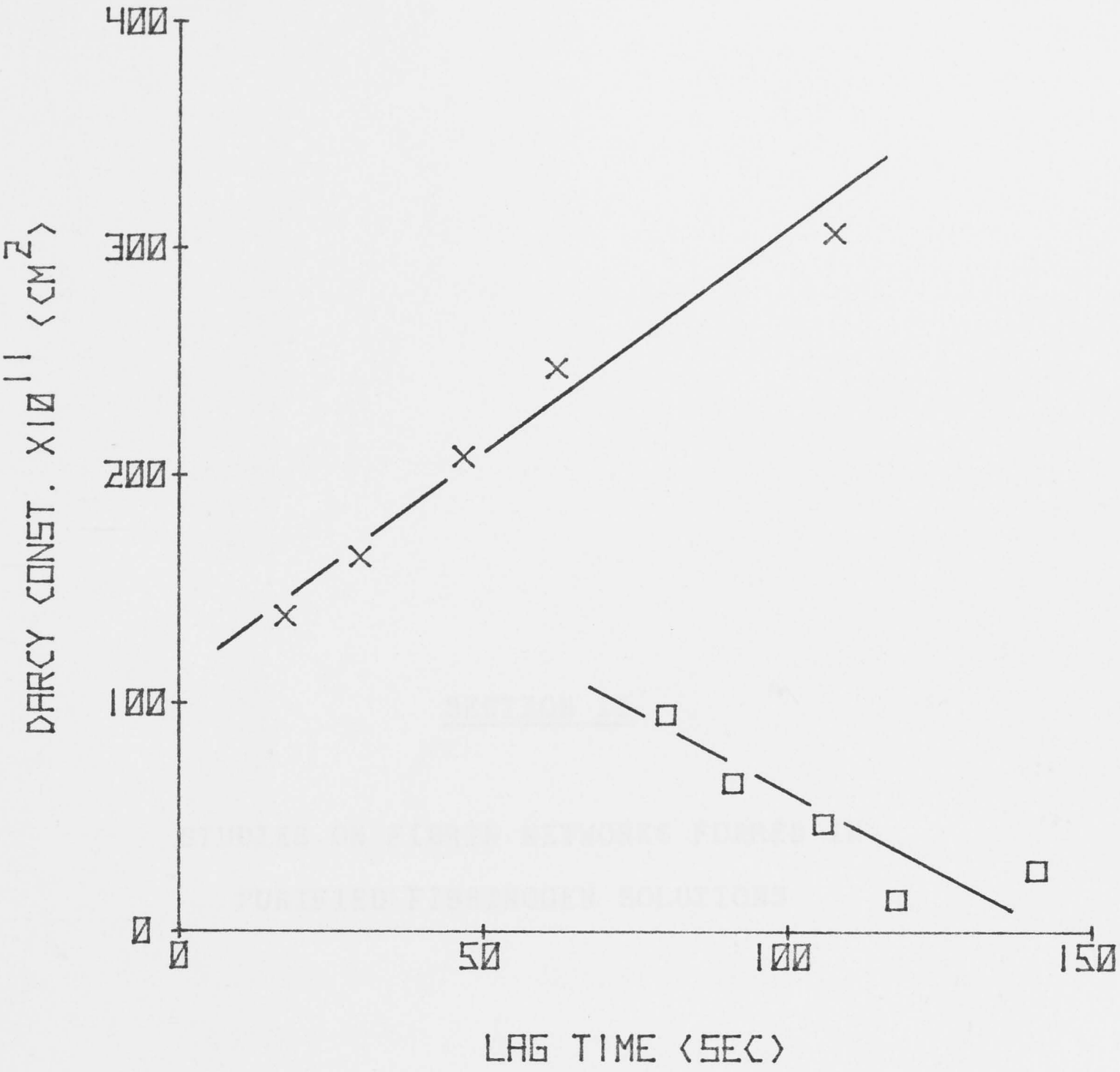


Figure 5.7 A plot of Darcy constant against lag time

Networks were formed in fibrinogen solution (3.3 mg/ml) without divalent cations. Ionic strength (□) or thrombin concentration (×) was varied (Chapter 6). Darcy constant is directly related ($r=0.999$, $p(F) < 0.0001$, $n = 5$) to lag time where thrombin concentration is varied and inversely related ($r= -0.873$, $p(F) < 0.05$, $n = 5$) where ionic strength is varied.

SECTION II

STUDIES ON FIBRIN NETWORKS FORMED IN PURIFIED FIBRINOGEN SOLUTIONS

CHAPTER 6

A RE-EXAMINATION OF SOME INFLUENCES ON FIBRIN NETWORK DEVELOPMENT, PROPERTIES AND STRUCTURE: STUDIES ON THE MECHANISM UNDERLYING CHANGES IN NETWORK STRUCTURE.

6.1 INTRODUCTION

Fibrin fibre diameter varies not only in different individuals (Chapter 9) in response to trauma and operation but even within a single thrombus (Hattori *et al*., 1978; Hisano, 1978) or haemostatic plug (Wester *et al*., 1978). Histological studies show that the variation in fibrin diameter within thrombi and haemostatic plugs is determined according to the location of fibrin in relationship to platelet mass. However, the mechanism underlying variation in fibre diameter observed under physiological conditions remains to be clarified.

Hermans and associates (Carr *et al*., 1977; Rosser et al., 1977, Carr and Hermans, 1978) and Wolfe and Waugh (1981) have shown that fibre thickness varies considerably *in vitro* when the ionic strength, pH, thrombin or fibrinogen concentrations are altered substantially. In these studies, however, the conditions of clotting were generally pharmacological rather than physiological. The relevance of previous observations to clotting *in vivo* remains unknown. In this Chapter factors already known to be of importance under pharmacological conditions of study were re-examined under conditions of clotting simulating those found in plasma.

6.2 MATERIALS AND METHODS

Unless otherwise indicated, fibrin networks were formed in fibrinogen solutions (3.3 mg/ml) which did not contain divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$, as described in Chapter 2. In addition, networks were made in fibrinogen solutions which contained 2.02 mM $MgCl_2$ to examine the effects of changes in ionic strength, pH and temperature. Turbidity, permeability and compaction were measured and used to calculate lag time, ΔOD/sec, opacity ratio, μ_T, μ_p and compaction (Chapter 2).

The effects of changes in ionic composition, ionic strength, pH, temperature and of changes in fibrinogen and thrombin concentration were explored as follows:

6.2.1 Effect of thrombin concentration

Networks were formed in different fibrinogen solutions by adding 0.05, 0.10, 0.15, 0.25 or 0.50 U/ml thrombin. To allow for the effect of thrombin concentration on the rate of fibrinogen conversion to fibrin, networks were left to develop for 90, 45, 30, 18 and 9 minutes, respectively. It was known from preliminary studies that at these times the amount of fibrinogen converted to fibrin was constant at the concentrations of thrombin examined.

6.2.2 Effect of fibrinogen concentration

Networks were formed in fibrinogen solutions which contained 1.0, 2.0, 3.3, 4.0, 6.0 and 8.0 mg/ml fibrinogen using 0.15 U/ml thrombin.

6.2.3 Effect of temperature

Networks were formed in fibrinogen solution at different temperatures which in each case was maintained constant until the networks were examined.

Fibrinogen solutions in tubes for permeation and compaction were pre-heated to 15°, 22°, 30°, 37° and 40°C by incubating in a water bath for 3 minutes to allow equilibration. Thrombin was then added to fibrinogen solutions. Networks were left to develop in the water bath at the appropriate temperature for 30 minutes before testing at room temperature.

With the turbidimetric technique, the lag time, ΔOD/sec, opacity ratio and μ_T were determined at the range of temperatures listed above and, in addition, at 20°, 25° and 35°C. Cuvettes containing fibrinogen solutions were placed in a thermostatically controlled holder for 4 minutes for equilibration and then thrombin was added. Readings of optical density at 350 and 608 nm were made at 5 minute intervals for 60 minutes.

6.2.4 Effects of ionic strength and pH

Networks were formed in fibrinogen solutions with ionic strengths of 0.133, 0.143, 0.153, 0.163 and 0.173 (ionic strength adjusted with NaCl) or in fibrinogen solutions with pH 7.15, 7.25, 7.35, 7.45 or 7.55.

6.2.5 Effect of common ions found in plasma

The effect of physiological concentrations of various ions on network opacity ratio and compaction were examined by clotting fibrinogen solution (3.3 mg/ml) in the presence of either 1.20 mM $CaCl_2$, 4.30 mM KCl, 0.82 mM $MgCl_2$, 1.11 mM NaH_2PO_4, 0.35 mM Na_2SO_4 or 29.00 mM $NaHCO_3$ (final concentrations). A range of concentrations of $CaCl_2$ and $MgCl_2$ were also examined. In all experiments, the ionic strength and pH of test solutions were kept constant. Solutions of NaH_2PO_4 and $NaHCO_3$ were adjusted to pH 7.35 immediately before use. These solutions were added to fibrinogen solution containing 100 mM Tris-HCl which provided superior pH control than the usual 50 mM Tris-HCl.

6.3 RESULTS

6.3.1 Effect of thrombin concentration

An increase in thrombin concentration decreased the lag time and enhanced ΔOD/sec (Figure 6.1). As shown, the lag

time correlated inversely with thrombin concentration and ΔOD/sec correlated linearly with thrombin concentration. Divalent cations decreased the lag time and increased ΔOD/sec at all thrombin concentrations.

An increase in thrombin concentration decreased both μ_T and μ_p (Figure 6.2). The inverse of thrombin concentration correlated linearly with both μ_T and μ_p. Divalent cations increased μ_T and μ_p at all thrombin concentrations.

In parallel with μ_T and μ_p, an increase in thrombin concentration resulted in a decrease in compaction, opacity ratio and permeability (Appendix 6). Network protein concentration increased only slightly with thrombin concentration.

6.3.2 Effect of fibrinogen concentration

An increase in fibrinogen concentration increased the lag time and ΔOD/sec (Figure 6.3). The lag time was linearly correlated with fibrinogen concentration. Divalent cations decreased the lag time and increased ΔOD/sec at all fibrinogen concentrations.

An increase in fibrinogen concentration generally decreased μ_T (Figure 6.4) and μ_p (Figure 6.5). In the absence of divalent cations, μ_T appeared not to change substantially at low concentrations of fibrinogen.

An increase in fibrinogen concentration increased opacity ratio (Figure 6.4) and decreased network compaction and permeability (Figure 6.5). In fact, Darcy constant was inversely correlated with the fibrinogen concentration (networks made without divalent cations : $r = -0.991$, $p(F) < 0.0001$; networks made with divalent cations : $r = -0.994$, $p(F) < 0.0001$).

6.3.3 Effect of temperature

The effect of temperature on the network growth rate was complex (Figure 6.6). The ΔOD/sec was maximal while the lag time was minimal at temperatures between 25° and 30°C in the absence of divalent cations. Changes in the lag time and ΔOD/sec were less distinct in the presence of divalent cations; the former increased above 35° while the latter decreased below 25°C.

Fibrinogen solutions which contained Ca^{++} gelled slowly when cooled below room temperature, even in the absence of thrombin. Gelling was most rapid at 15°C.

An increase in temperature caused a non-linear increase in μ_T and μ_p (Figure 6.7). The changes in μ_T and μ_p with temperature paralleled the changes in compaction, opacity ratio and permeability (Appendix 6). The network protein concentration decreased only slightly with temperature.

6.3.4 Effects of ionic strength and pH

An increase in ionic strength resulted in a decrease in ΔOD/sec and a less distinct increase in the lag time (Figure 6.8). Divalent cations (Ca^{++} > Mg^{++}) decreased the lag time and increased ΔOD/sec.

An increase in ionic strength (Fig. 6.9) or in pH (Figure 6.10) decreased μ_T and μ_p. These changes paralleled the changes in compaction, opacity ratio and permeability (Appendix 6). Network protein concentration increased only slightly with ionic strength or pH.

6.3.5 Effect of common plasma ions

With the exception of Ca^{++} and Mg^{++}, physiological concentrations of $SO_4^{=}$, HPO_4^{-}, HCO_3^{-} and K^{+} did not significantly influence network opacity ratio of compaction (Table 6.1). Ca^{++} and Mg^{++} induced similar increases in opacity ratio and, at divalent cation concentrations below 5.0 mM, both induced similar increases in network compaction (Figure 6.11). At higher divalent cation concentrations, however, networks made in the presence of Ca^{++} showed less compaction than those made in the presence of Mg^{++}.

6.4 DISCUSSION

Previous studies on the effect of ionic strength, pH,

temperature, thrombin and fibrinogen concentration (Carr et al., 1977; Rosser et al., 1977; Carr and Hermans, 1978; Wolfe and Waugh, 1981) have tended to concentrate on the mechanics of polymerization rather than on the effect of these variables on fibrin network properties under physiological conditions of clotting. As a result often a single technique was used. The findings from such studies have not provided conclusive evidence that the consistent variation seen in fibre thickness in normal haemostatic plugs and thrombi (Hattori et al., 1978; Hisano, 1978; Wester et al., 1978) has its origins in local clotting conditions found in vivo. In the studies described in this Chapter particular attention was paid to examine fibrin network characteristics under relatively physiological conditions of clotting in which fibrinogen and thrombin concentrations, pH, ionic strength, ionic composition and temperature were varied in the range expected in vivo.

In addition a variety of techniques were used to examine fibrin network characteristics to allow methodological difficulties (Chapters 3, 4 and 5) to be overcome. By so doing it was possible to make several interesting observations. For example, it was observed that μ_T and μ_p generally change in parallel. Except when fibrinogen concentration is varied, the opacity ratio, permeability and compaction generally parallel the mass-length ratio.

Opacity ratio and compaction, however, did not change

in parallel where divalent cation concentration was increased (Figure 6.11) and as discussed in Chapter 4, this Ca^{++} specific effect on compaction may be attributed to stabilization of the minor network caused by factor $XIII_a$ mediated crosslinking in the presence of Ca^{++}. Although minor differences between the effects of Ca^{++} on network structure may derive from conformational changes in the fibrin(ogen) molecule due its Ca^{++} specific binding sites (Marguerie et al., 1977), it is notable that Mg^{++} and Ca^{++} increased opacity ratio in an identical manner (Figure 6.11). Furthermore, the presence of divalent cations did not influence the response of fibrin networks to other changes in clotting conditions. These findings indicate, firstly, that ionic charge is important in influencing network structure and secondly, that factor $XIII_a$ mediated crosslinking does not greatly affect the polymerization mechanism and the resultant network structure.

It is not clear how changes in clotting conditions lead to changes in fibre thickness. Ferry and Morrison (1947) suggested that thin fibres form when the solubility of fibrin(ogen) is great since an increase in solubility reduces the tendency for fibrin monomers to aggregate. Consequently, an increase in ionic strength or pH which leads to increased solubility (Ferry and Morrison, 1947) promotes smaller mass-length ratio (Figures 6.9 and 6.10, respectively). In keeping with this suggestion the lag time increases as ionic strength increases (Figure 6.8;

Hantgan and Hermans, 1979). However this mechanism fails to explain the relationship between fibre thickness and fibrinogen or thrombin concentration described in this Chapter. Increase in fibrinogen or thrombin concentration reduces the mass-length ratio (Figures 6.2, 6.4 and 6.5) without affecting fibrin(ogen) solubility.

More recently Nelb et al. (1976) have proposed that fibrin networks are metastable structures in which fibres diffuse through solvent and associate laterally with their neighbours, except when movement is prevented by their neighbours. According to this suggestion the time available for fibril diffusion becomes a critical factor. Fibre thickness will be determined by the density and rate of development of the network. An increase in the rate of fibrin generation induced by increase in thrombin concentration induces a lower mass-length ratio (Figure 6.2) because it reduces the extent of realignment of fibrils into thicker fibres.

An increase in fibrogen concentration results in a decrease in mass-length ratio (Figures 6.4 and 6.5) and an increase in fibril density. The extent of realignment of fibrils into thicker fibres decreases at physiological concentrations of fibrinogen but not in dilute fibrin gels (below 1 mg/ml) in which μ_T has previously been reported to increase with fibrinogen concentration (Carr et al., 1977; Hantgan and Hermans, 1979; Wolfe and Waugh, 1981). Rosser et al. (1977) suggest that at very low fibrin

concentrations the density of fibres in the network does not limit the extent of realignment of fibrils into thick fibres.

Temperature influences fibrin fibre thickness in a complex way. It affects the kinetics of fibrin generation through affecting thrombin activity. It also influences solubility of fibrin(ogen) as evidenced by the gelation of fibrinogen solution at low temperatures. The combined effects of temperature on the kinetics of fibrin generation and on the solubility of fibrin(ogen) should account for the increase in mass-length ratio with temperature (Figure 6.7) as well as the changes in the lag time and ΔOD/sec (Figure 6.8).

It has been shown in this Chapter that the thickness of fibrin fibres and network properties are sensitive to small changes in clotting conditions. Shifts in ionic strength, pH and temperature within the ranges normally found in plasma result in considerable changes in mass-length ratio and in network properties. Substantial differences in network properties are also induced by varying fibrinogen and thrombin concentrations. These findings suggest that if these parameters change *in vivo* the fibrin network structure may be expected to vary.

The thickness of fibrin fibres in thrombi is known to vary consistently and predictably (Hattori *et al.*, 1978; Hisano, 1978). But the mechanism underlying this variation

remains unclear. It has been suggested that compaction of thinner fibres into thicker fibre bundles during platelet mediated clot retraction accounts for the variation (Szalontai, 1968). The results of this study suggest that local differences in the clotting conditions may, in addition, be responsible for variation in fibre thickness in thrombi.

In circulating plasma the ionic strength, pH and temperature are normally tightly controlled within narrow limits (White et al., 1973). Fibrin, however, normally forms at the site of injury where alterations may be expected in pH, temperature and possibly ionic strength. In such regions, fibre thickness and clot properties will probably reflect the local ionic strength, pH and temperature.

Although ionic strength, pH and temperature are tightly controlled in plasma, thrombin and fibrinogen concentration vary greatly. Thrombin is not present in appreciable amounts in plasma but during coagulation its concentration may exceed 10 U/ml (Lui et al., 1979). Fibrinogen is an acute phase reactant and its plasma concentration can increase from 2-4 mg/ml in health to greater than 7 mg/ml in some inflammatory states (Johansson, 1976; Chapter 8). Further, during local inflammation, oedema fluid contains very high concentrations of many plasma proteins including fibrinogen; this fluid sometimes clots spontaneously (Walter and Israel, 1974). Studies in this Chapter show

that fluctuations in thrombin and fibrinogen concentration not only affect the intrinsic mass of the clot and how quickly it forms, but also the structure and properties of the fibrin. Therefore, it might be expected that similar fluctuations in thrombin and fibrinogen concentration *in vivo* will also affect fibrin structure and properties.

6.5 CONCLUSIONS

(1) Fibrin fibre thickness is sensitive to small changes in clotting conditions within the ranges found in plasma.

(2) Divalent cations and factor $XIII_a$ mediated cross-linking do not substantially influence the changes in fibre thickness induced by any one of a variety of changes in clotting conditions.

(3) Results indicate that small alterations in the ionic strength, pH or temperature or in the thrombin or fibrinogen concentration of plasma will probably affect fibrin network stucture and properties.

Table 6.1 The effect of common ions found in plasma on compaction and opacity ratio

	OPACITY RATIO	% COMPACTION
Control	0.432 ± 0.008	7.2 ± 0.5
4.30 mM KCl	0.432 ± 0.009	7.0 ± 0.9
0.35 mM Na_2SO_4	0.436 ± 0.007	6.8 ± 0.4
1.20 mM $CaCl_2$	0.453 ± 0.016*	8.1 ± 0.7*
0.82 mM $MgCl_2$	0.454 ± 0.009*	7.9 ± 1.0*
Control	0.422 ± 0.011	6.8 ± 0.8
1.11 mM NaH_2PO_4	0.422 ± 0.001	6.0 ± 1.0
29.00 mM $NaHCo_3$	0.412 ± 0.010	6.0 ± 0.7

Networks were formed in fibrinogen solution (3.3 mg/ml) to which various salts were added. Networks formed in the presence of NaH_2PO_4 and $NaHCO_3$ contained 100 mM Tris-HCl. Results are the mean of 9 determinations ± SD. * indicates values significantly different ($p(F)<0.01$) from controls.

Figure 6.1 The effect of thrombin concentration on lag time and ΔOD/sec

The lag time correlated inversely with thrombin concentration (networks made without divalent cations : $r = -0.999$, $p(F) < 0.0001$; networks made with divalent cations $r = -0.997$, $p(F) < 0.0001$).

The ΔOD/sec correlated linearly with thrombin concentration (networks made without divalent cations : $r = 0.999$, $p(F) < 0.0001$; networks made with divalent cations : $r = 0.999$, $p(F) < 0.0001$).

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 3 determinations.

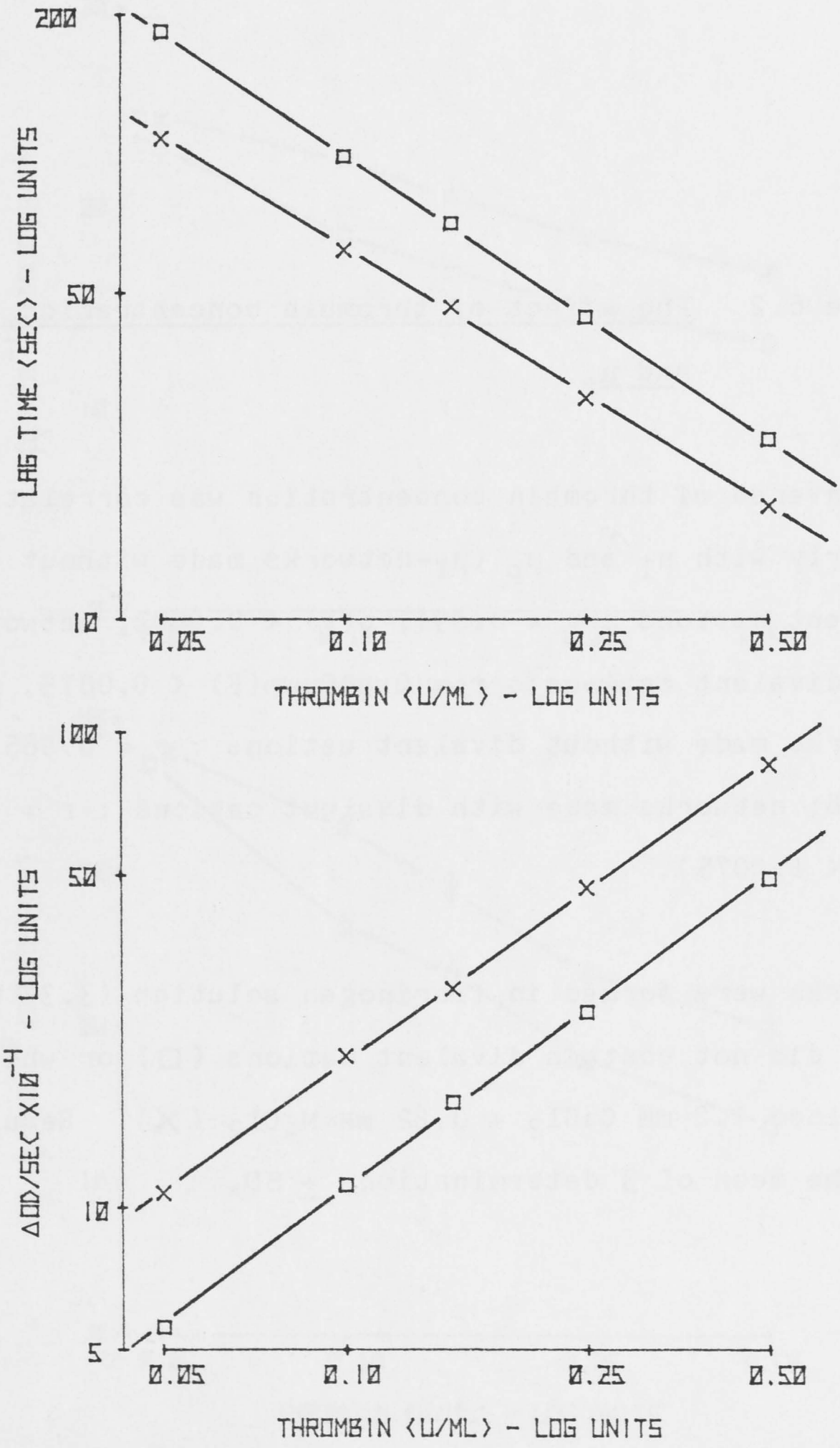
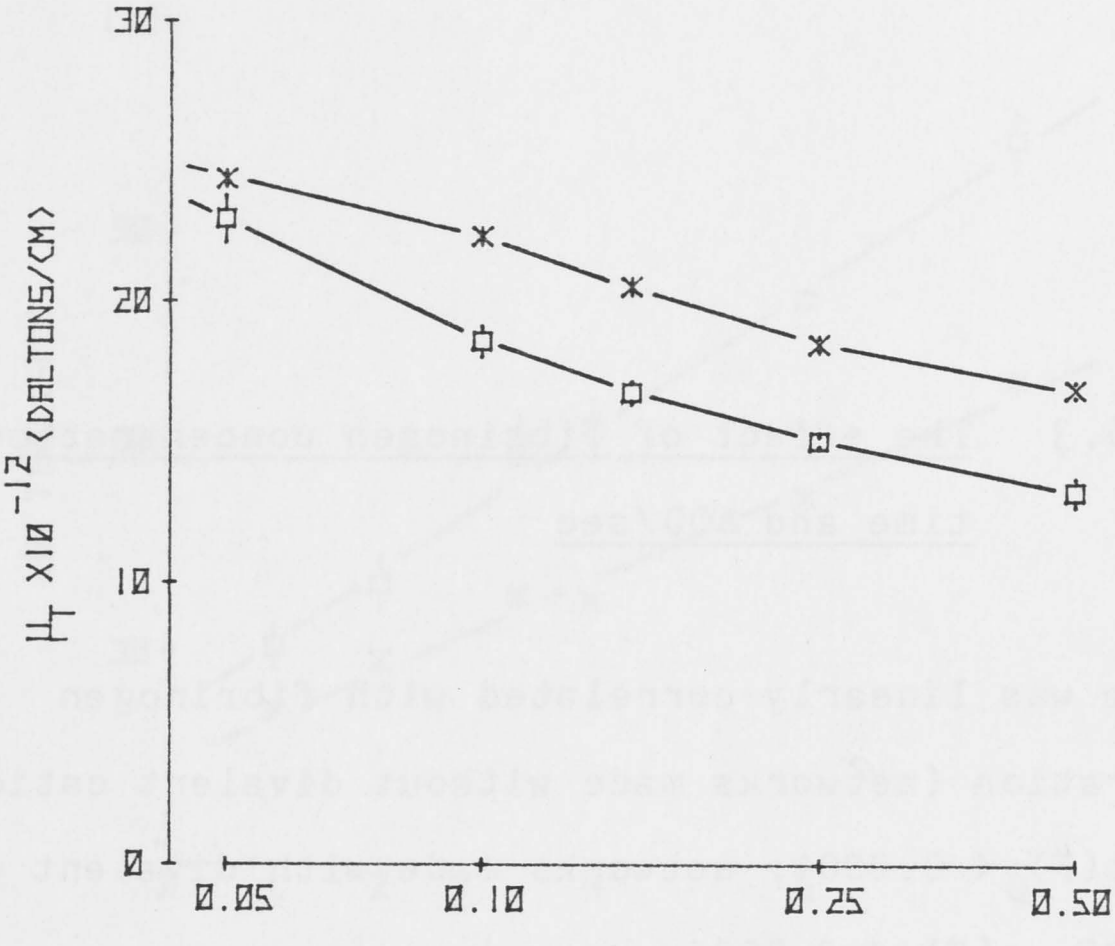

200
50
10
LAG TIME (SEC) - LOG UNITS
0.05
0.10
0.25
0.50
THROMBIN (U/ML) - LOG UNITS
100
50
10
5
ΔOD/SEC X10^{-4} - LOG UNITS
0.05
0.10
0.25
0.50
THROMBIN (U/ML) - LOG UNITS

Figure 6.2 The effect of thrombin concentration on μ_T and μ_p

The inverse of thrombin concentration was correlated linearly with μ_T and μ_p (μ_T-networks made without divalent cations : r = 0.991, p(F) < 0.0002; networks made with divalent cations : r = 0.945, p(F) < 0.0075. μ_p-networks made without divalent cations : r = 0.985, p(F) < 0.0006; networks made with divalent cations : r = 0.944; p(F) < 0.0075).

Networks were formed in fibrinogen solution (3.3. mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 determinations ± SD.


30
20
10
0
μ_T X10^{-12} (DALTONS/CM)
0.05
0.10
0.25
0.50
THROMBIN (U/ML) - LOG UNITS

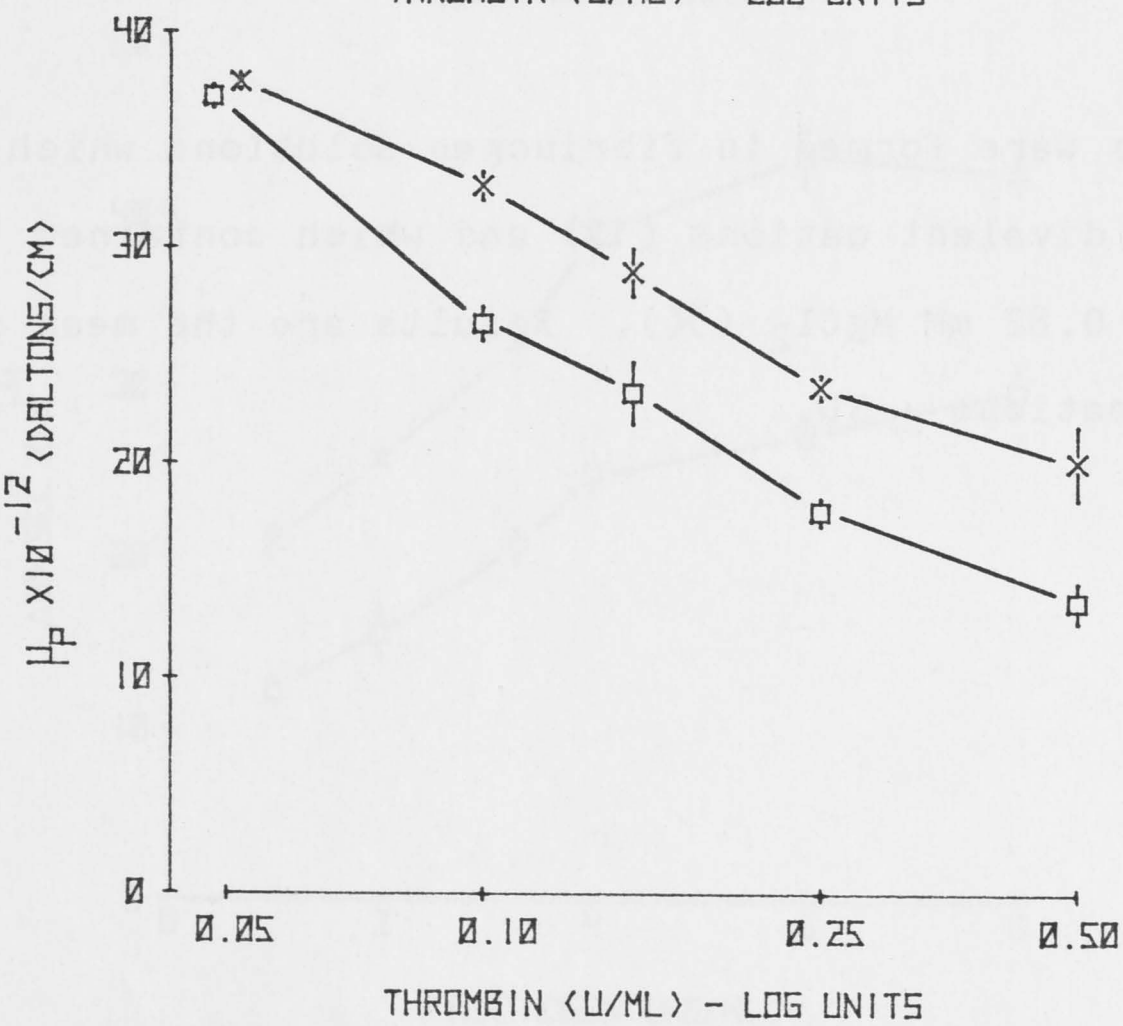
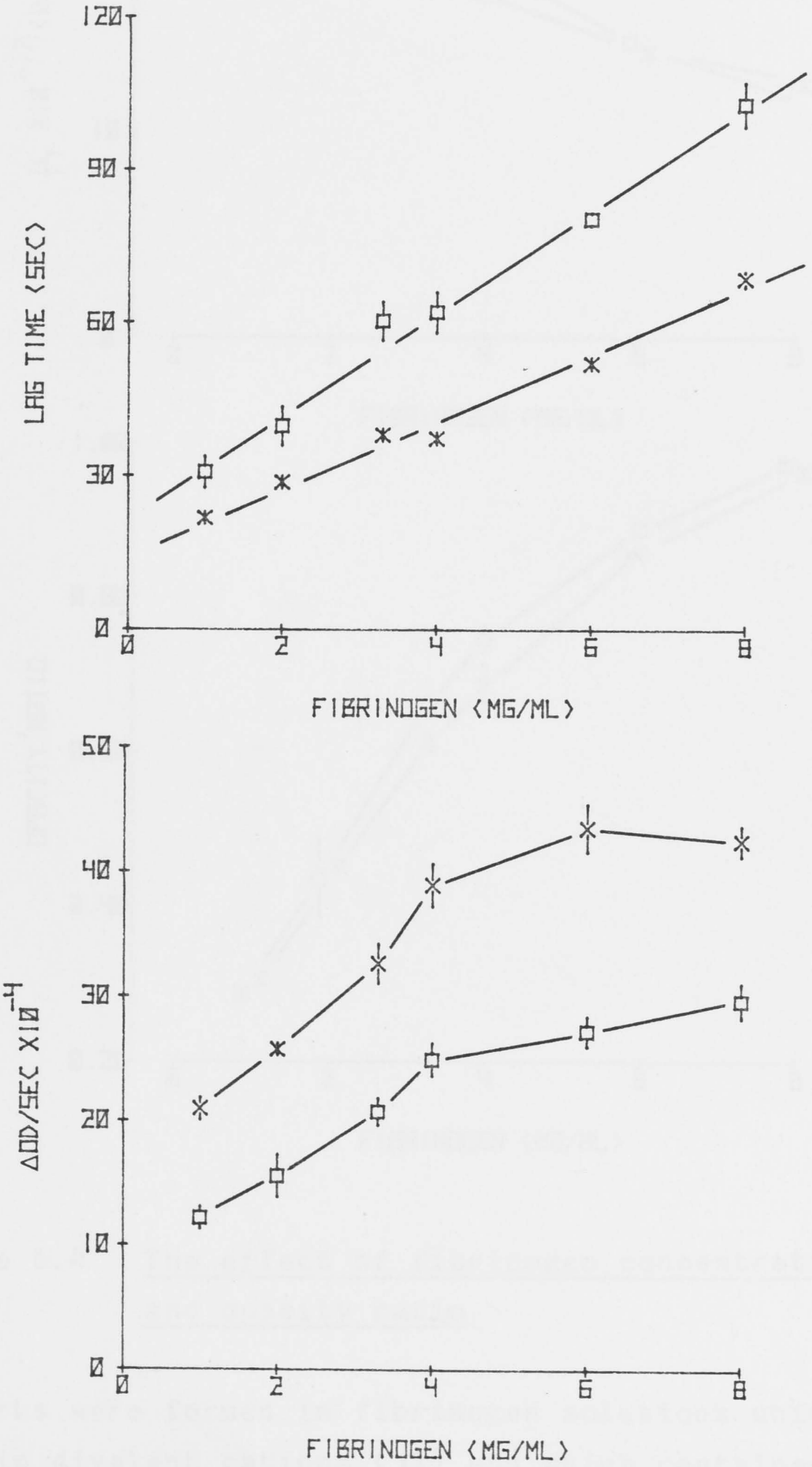

40
30
20
10
0
μ_P X10^{-12} (DALTONS/CM)
0.05
0.10
0.25
0.50
THROMBIN (U/ML) - LOG UNITS

Figure 6.3 The effect of fibrinogen concentration on lag time and ΔOD/sec

Lag time was linearly correlated with fibrinogen concentration (networks made without divalent cations : $r = 0.994$, $p(F) < 0.0001$; networks made with divalent cations : $r = 0.992$, $p(F) < 0.0001$.

Networks were formed in fibrinogen solutions which did not contain divalent cations (□) and which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 determinations ± SD.


120
90
60
30
0
LAG TIME (SEC)
0
2
4
6
8
FIBRINOGEN (MG/ML)
50
40
30
20
10
0
ΔOD/SEC X10⁻⁴
0
2
4
6
8
FIBRINOGEN (MG/ML)

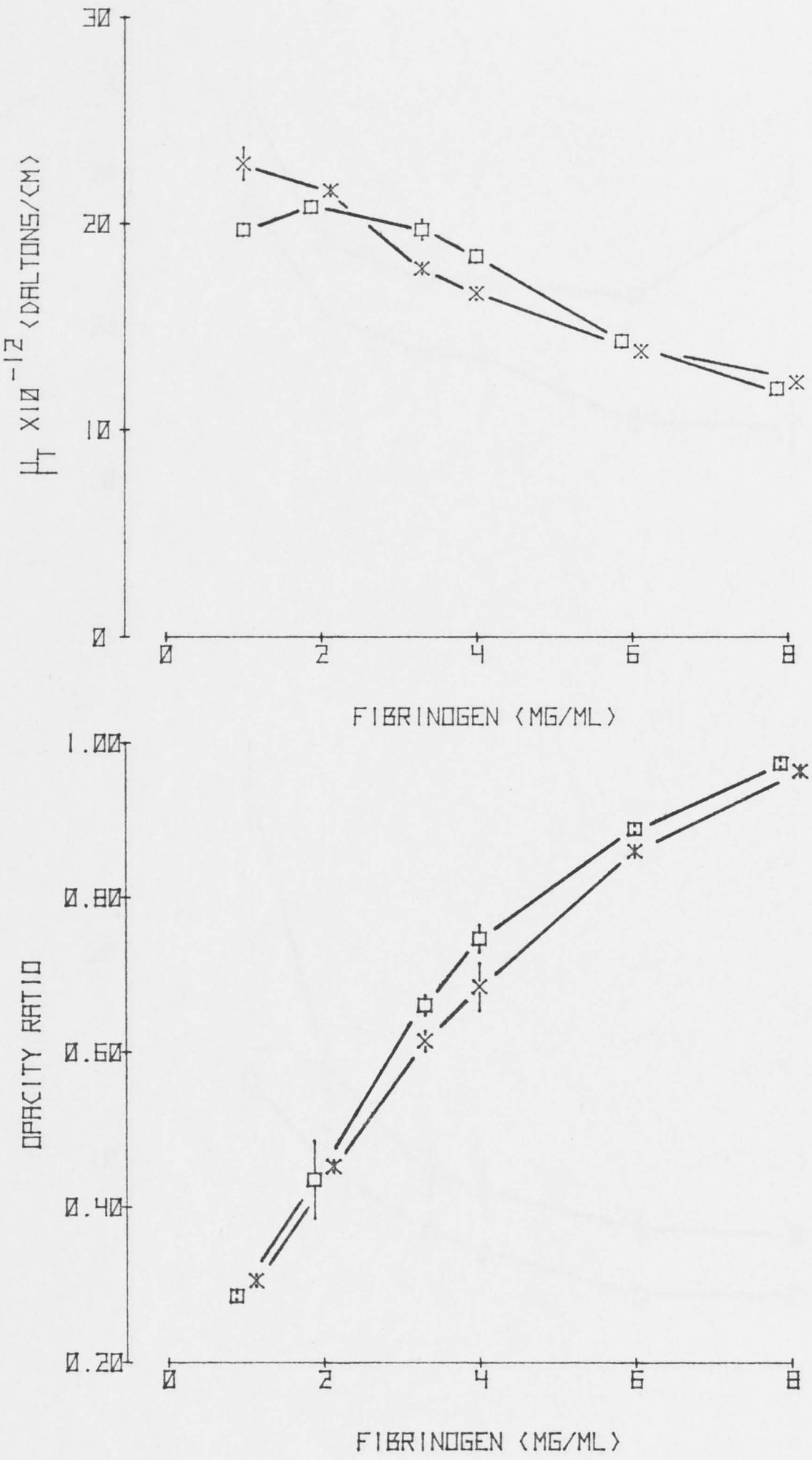


Figure 6.4 The effect of fibrinogen concentration on μ_T and opacity ratio

Networks were formed in fibrinogen solutions which did not contain divalent cations (□) and which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 determinations ± S.D.

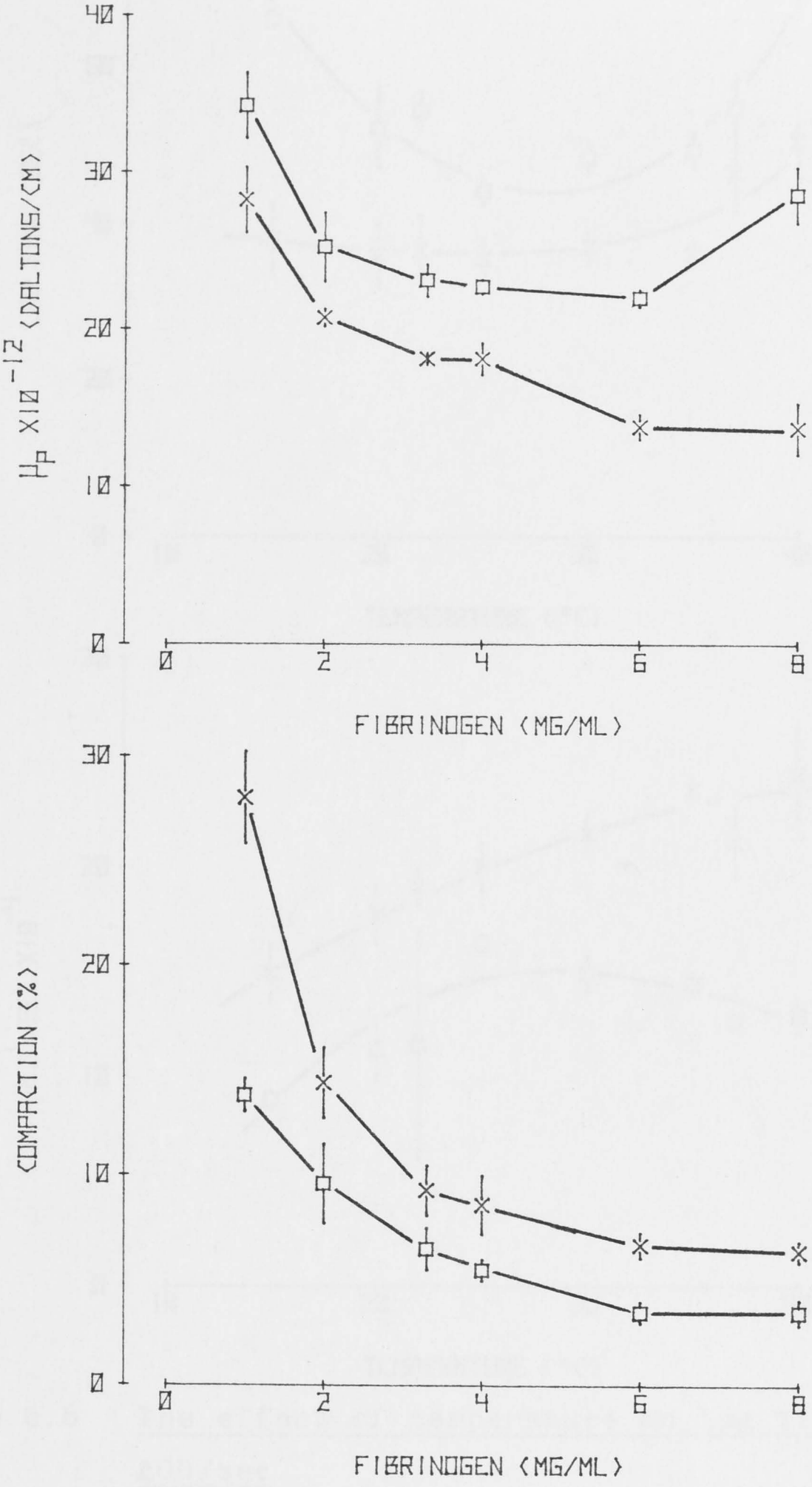
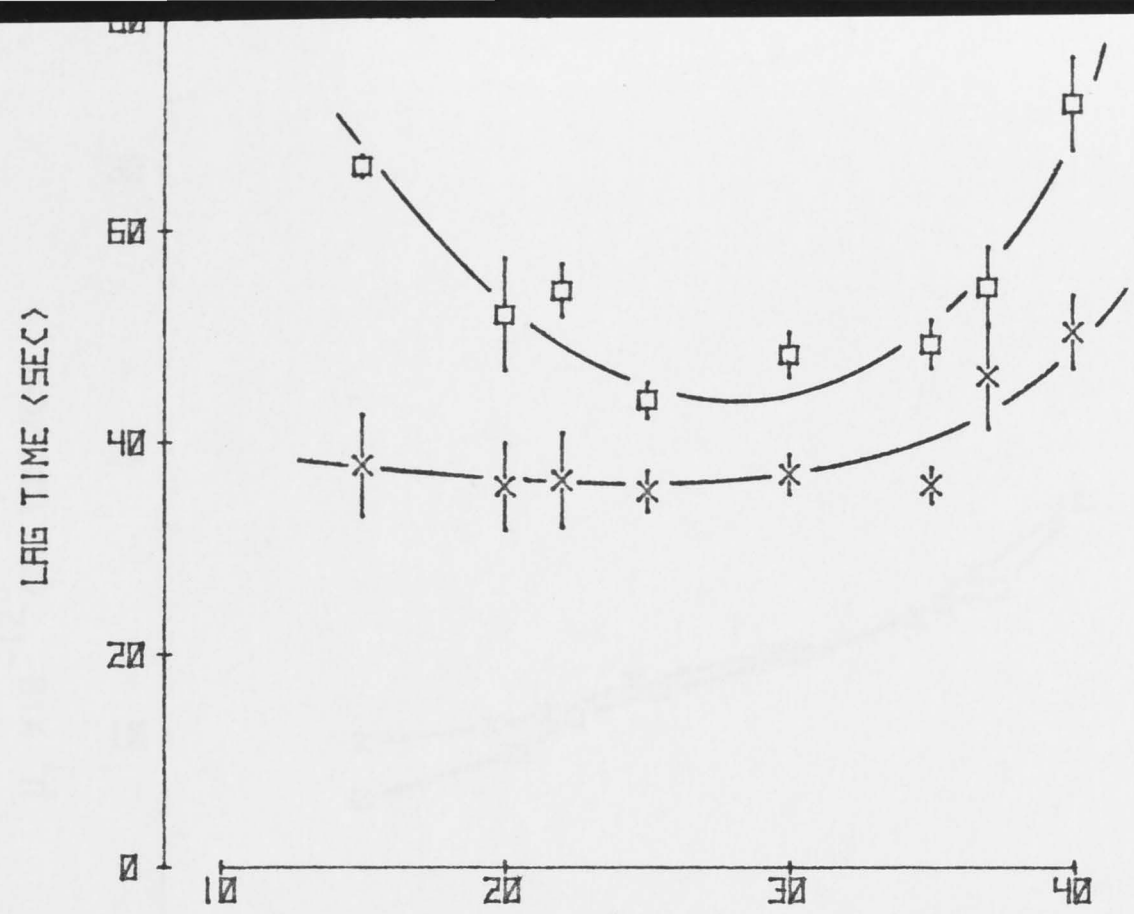


Figure 6.5 The effect of fibrinogen concentration on μ_p and network compaction

Networks were formed in fibrinogen solutions which did not contain divalent cations (□) and which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 3 determinations ± SD.



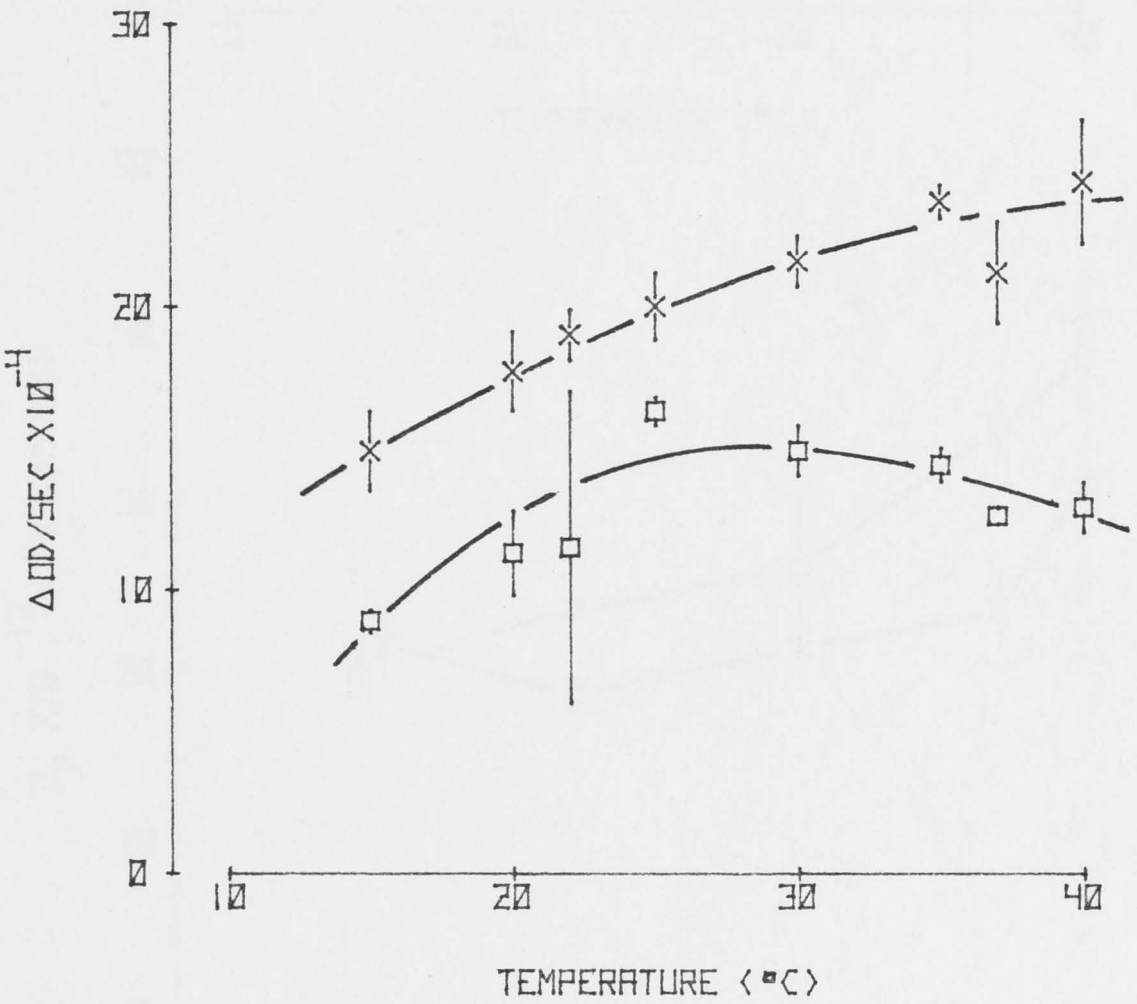


Figure 6.6 The effect of temperature on lag time and ΔOD/sec

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 5 determinations ± SD. For graphic simplicity data from networks formed in the presence of 2.02 mM $MgCl_2$ is not shown as it falls midway between the other two sets of data.

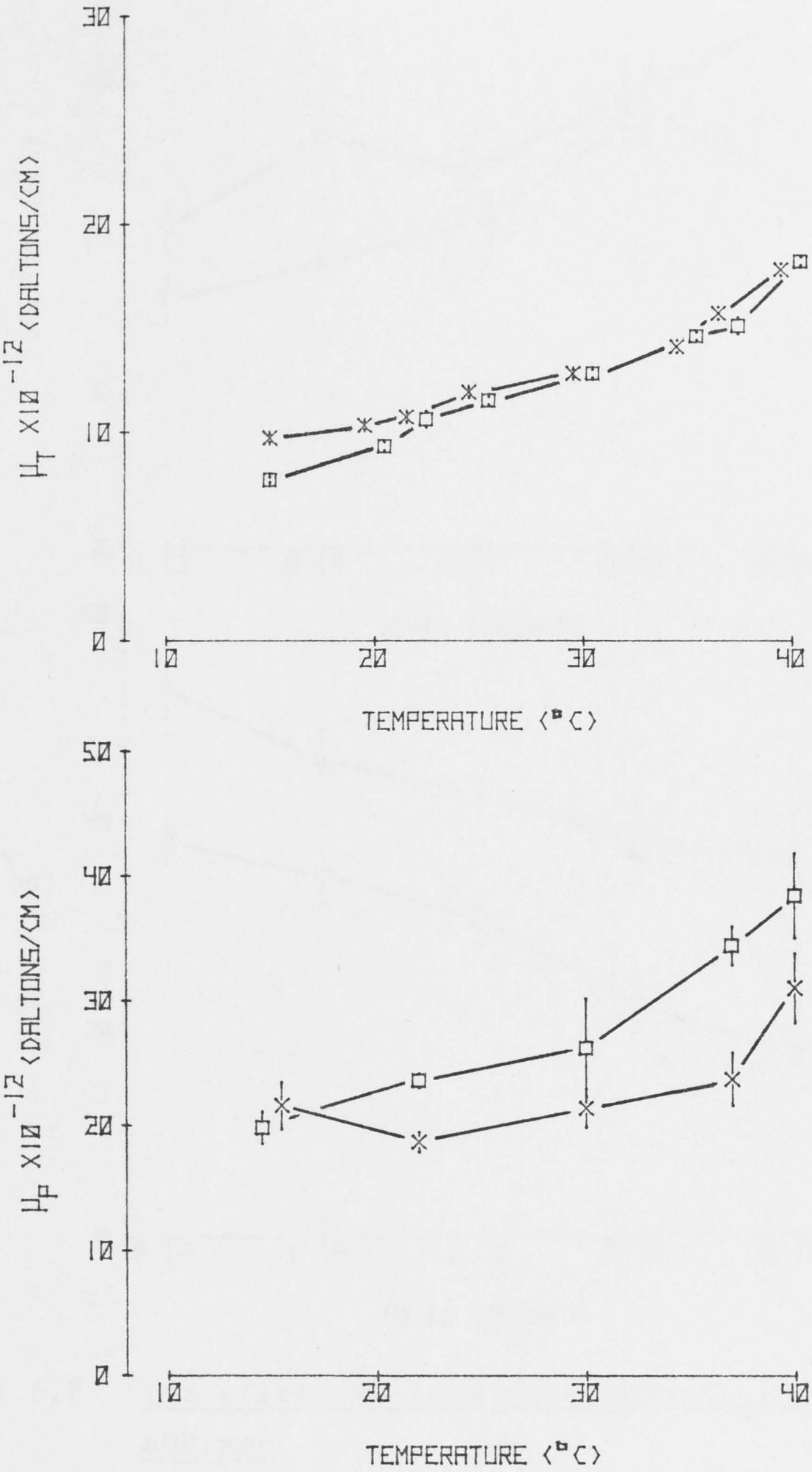


Figure 6.7 The effect of temperature on μ_T and μ_p

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 5 determinations ± SD. For graphic simplicity data from networks formed in the presence of 2.02 mM $MgCl_2$ is not shown.

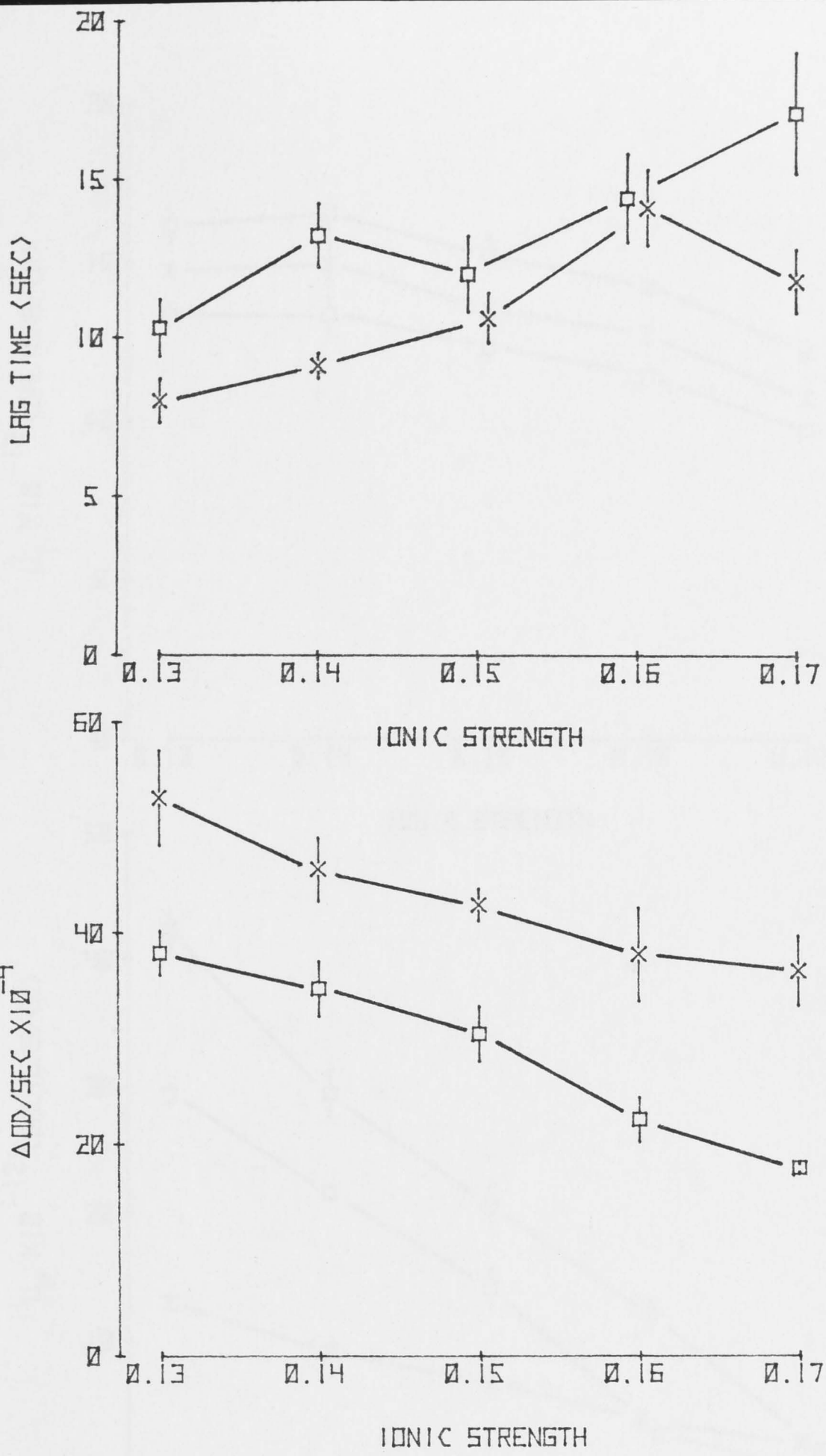


Figure 6.8 The effect of ionic strength on lag time and ΔOD/sec

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 5 determinations ± SD. For graphic simplicity data from networks formed in the presence of 2.02 mM $MgCl_2$ is not shown. It falls midway between the other two sets of data.

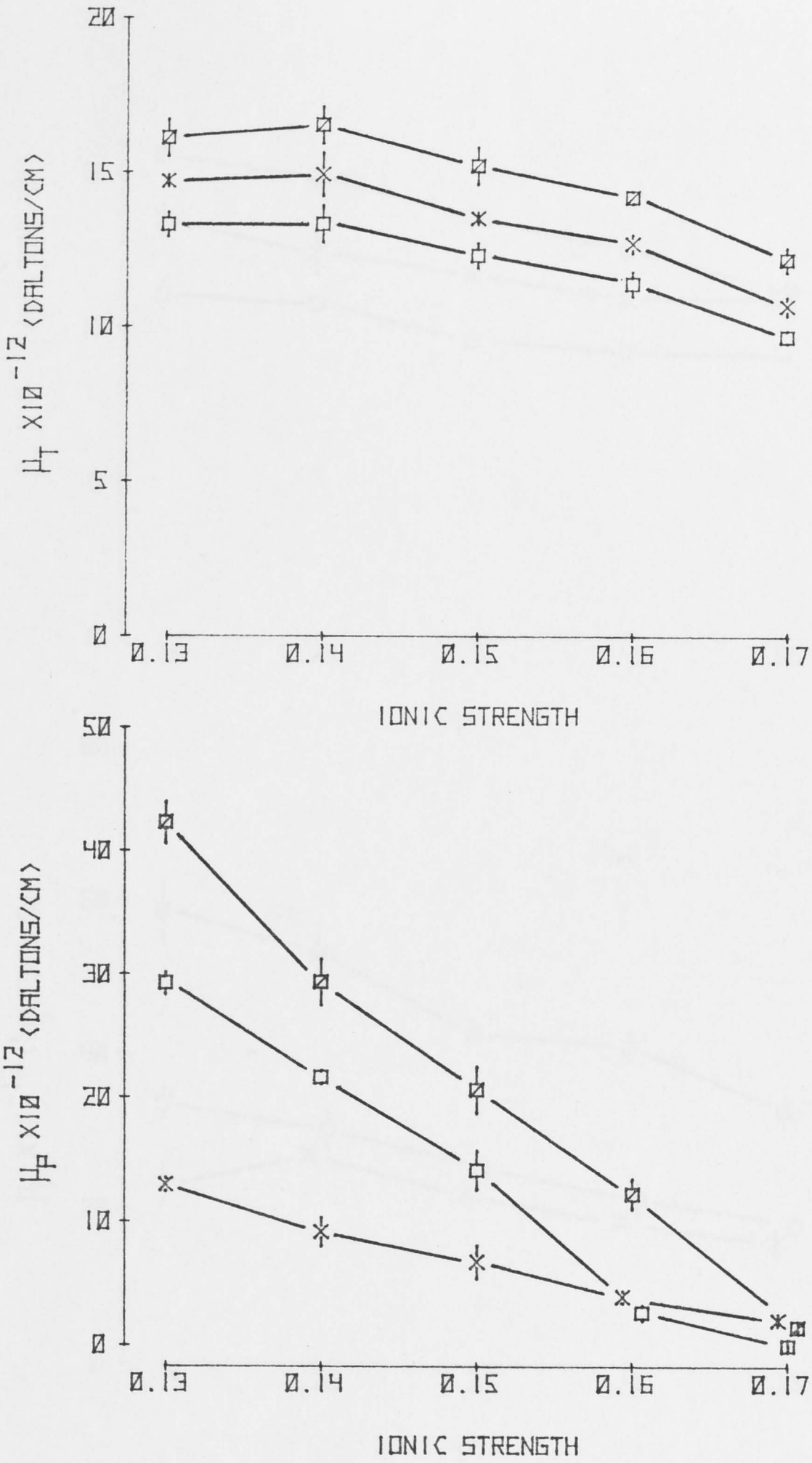


Figure 6.9 The effect of ionic strength on μ_T and μ_p

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□), which contained 2.02 mM $MgCl_2$ (⊠) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 5 determinations ± SD.

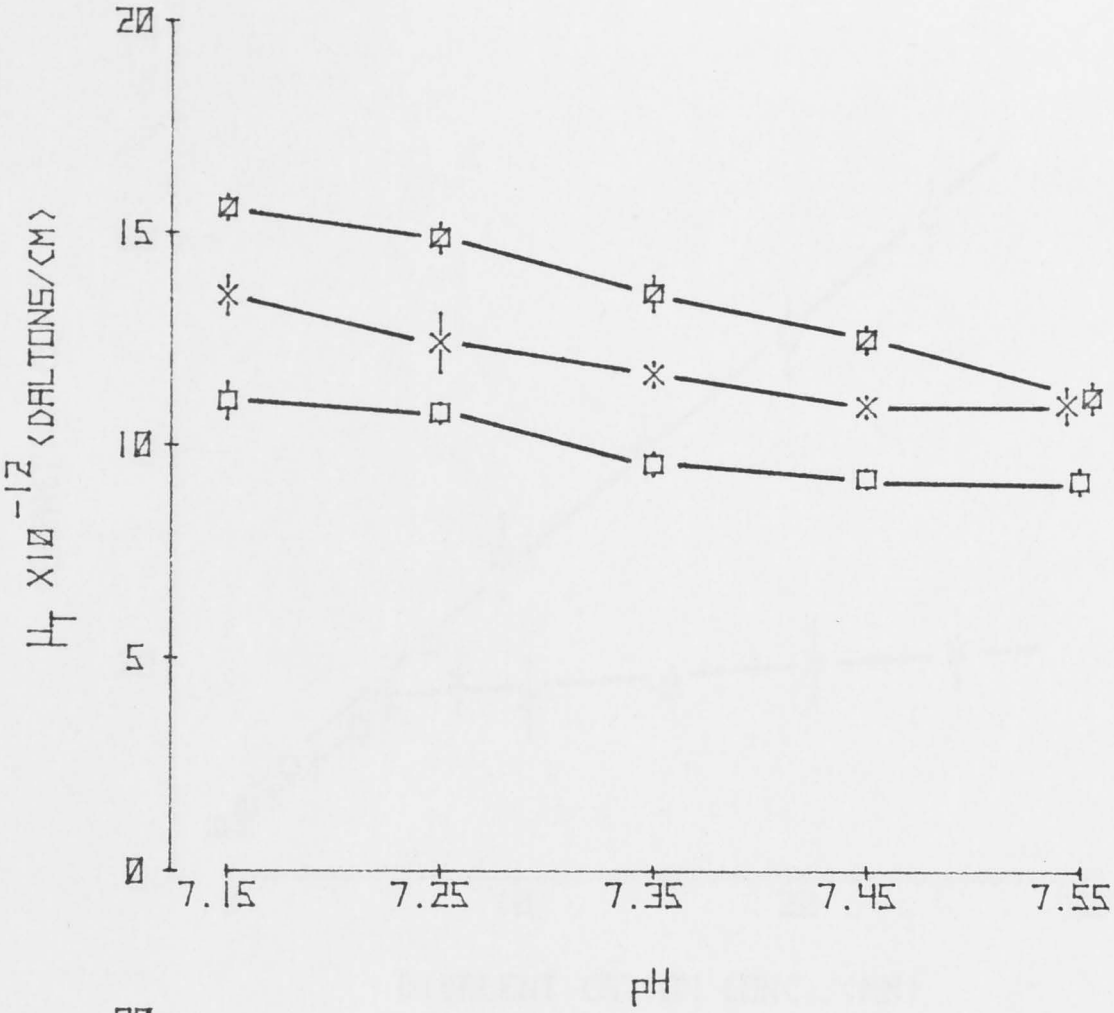
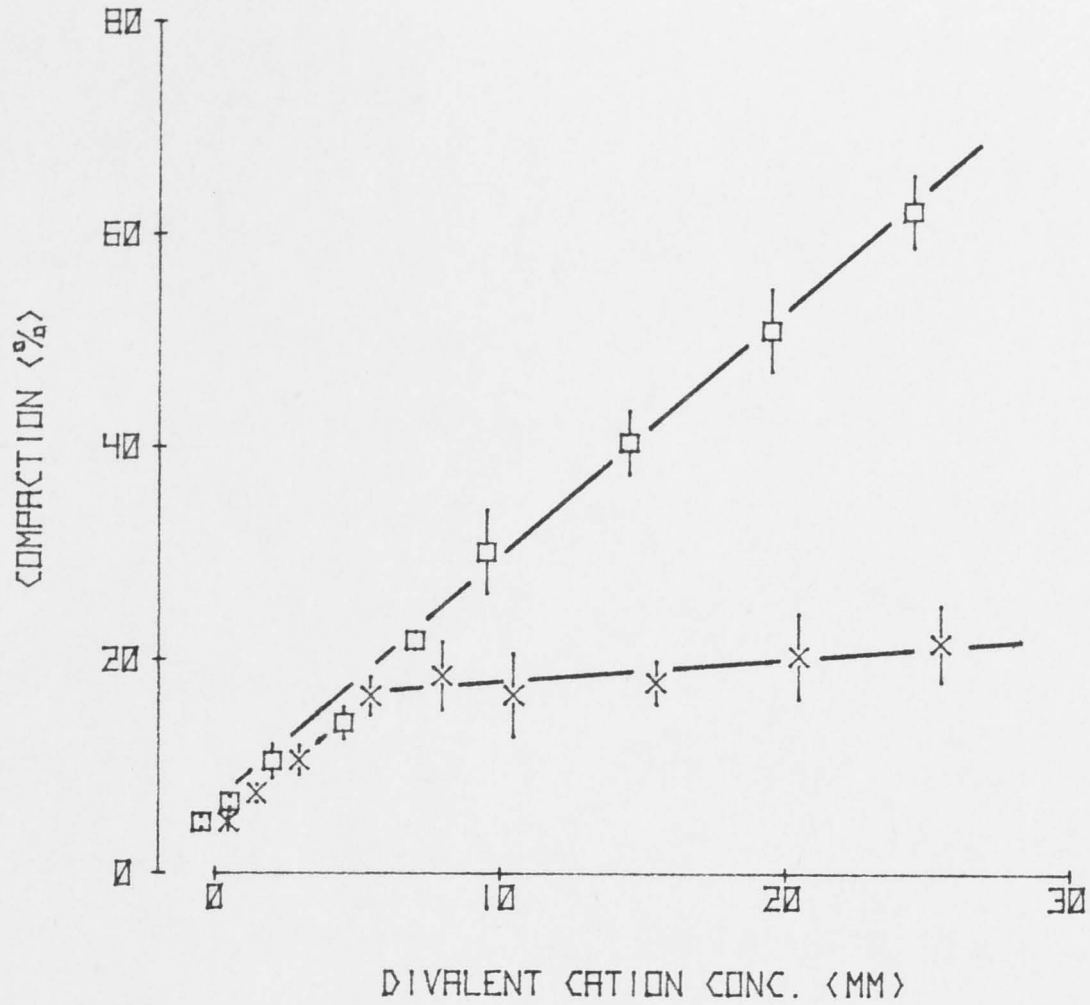


Figure 6.10 The effect of pH on μ_T, and μ_p

Networks were formed in fibrinogen solution (3.3 mg/ml) which did not contain divalent cations (□), which contained 2.02 mM $MgCl_2$ (⊠) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 5 determinations ± SD.



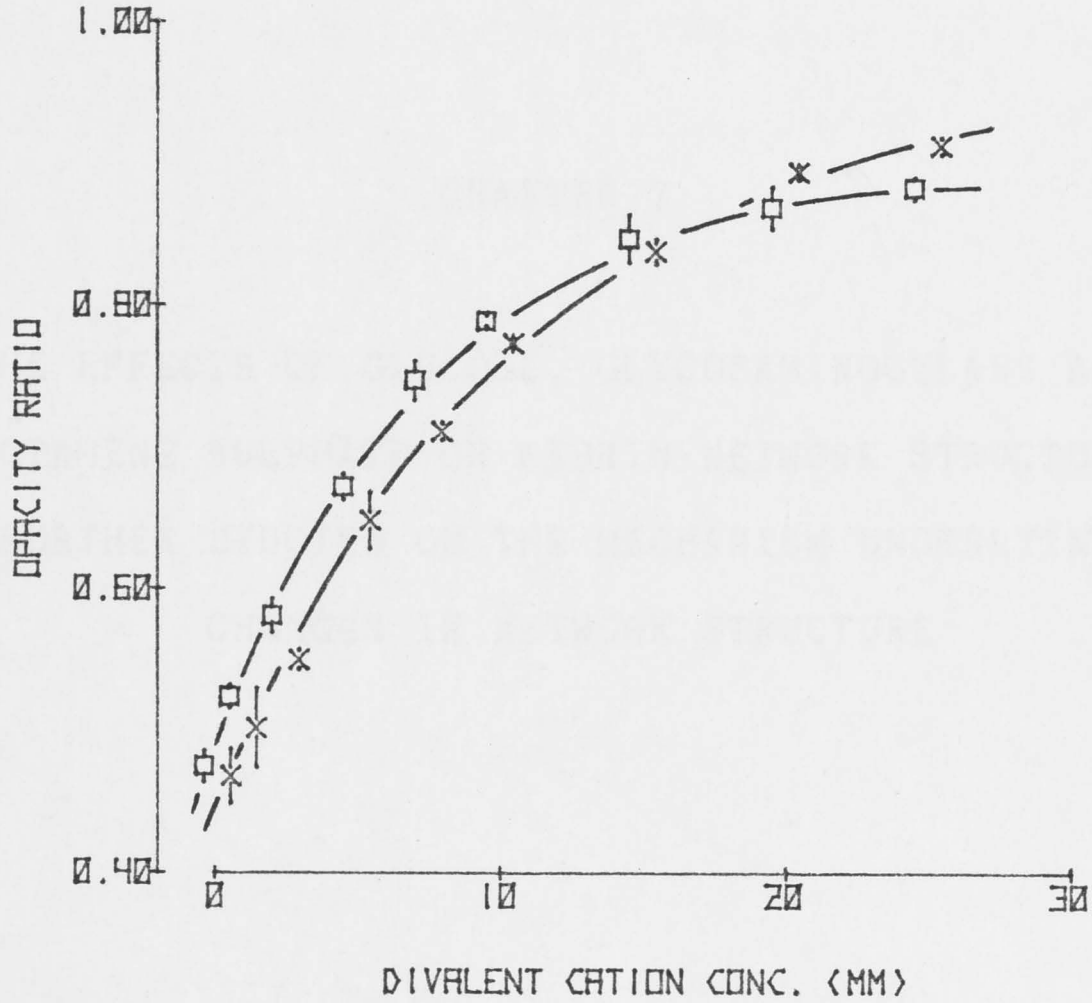


Figure 6.11. The effect of Ca^{++} and Mg^{++} on opacity ratio and compaction

Networks were formed in fibrinogen solution (3.3. mg/ml) which contained a range of divalent cation concentrations; $CaCl_2$ (×), $MgCl_2$ (□). Results are the mean of 5 determinations ± SD.

CHAPTER 7

THE EFFECTS OF GLUCOSE, GLYCOSAMINOGYLANS AND PROTAMINE SULPHATE ON FIBRIN NETWORK STRUCTURE: FURTHER STUDIES ON THE MECHANISM UNDERLYING CHANGES IN NETWORK STRUCTURE

7.1 INTRODUCTION

Plasma is a complex solution which contains many solutes besides fibrinogen. Influence on fibrin network structure of constituents other than fibrinogen is largely unknown. It is known that some substances, such as glucose (Ferry and Morrison, 1947), heparin (Sheppard et al., 1957), protamine sulphate (Carlin et al., 1976) and dextran (Muzaffar, 1974), may modify fibrin network properties under pharmacological conditions. It was shown in the last Chapter that networks are sensitive to small changes in clotting conditions. It seems reasonable to expect that these agents may also affect fibrin network structure under clotting conditions similar to those in plasma.

In this Chapter, a variety of physiologically and clinically important substances known to be sometimes present during fibrin formation in plasma are examined.

7.2 MATERIALS AND METHODS

Unless otherwise stated fibrin networks were formed in fibrinogen solutions which did not contain any added divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (Chapter 2). Networks were made in the presence of a variety of substances as described below. Except where otherwise indicated, μ_T, μ_p, network protein concentration, opacity ratio, compaction and permeability were determined as described in Chapter 2.

7.2.1 Effect of glucose

Glucose was added to fibrinogen solution (3.3 mg/ml) to give final concentrations of 10, 40, 70 and 100 mM. In other experiments, either 12.5, 20, 35, or 50 mM NaCl was added to fibrinogen solutions to adjust the osmolarity to that of the solutions which contained glucose.

In another set of experiments, either 25, 50, 75 or 100 mM glucose and ^{125}I-labelled fibrinogen were added to the fibrinogen solution (1.5 mg/ml). Clotting was initiated with 1.5 U/ml thrombin. Networks were left to develop fully for 1 hour and then perfused. The radioactivity and absorbance at 282 nm of the perfusate were then determined (Chapter 2).

7.2.2 Effect of heparin

Mucous sodium heparin (David Bull Laboratories) was added to fibrinogen solutions (3.3 mg/ml and 1.5 mg/ml) to yield final concentrations of 0.18, 0.90, 1.80, 9.0 or 18.0 U/ml. Networks formed in these solutions were examined for compaction, permeability, network protein concentration and μ_p. These networks and others formed in the presence of 0.5 U/ml heparin were also examined for opacity ratio and μ_T.

In addition, networks were formed in fibrinogen

solution (2.50 and 5.0 mg/ml) which contained (i) 0.5 U/ml heparin, (ii) 1 µg/ml protamine sulphate or (iii) 0.5 U/ml heparin + 1 µg/ml protamine sulphate. The mass-length ratios were calculated from turbidity measurements.

7.2.3 Effect of protamine sulphate

Protamine sulphate (Sigma, U.S.A.) alone precipitated fibrinogen at concentrations greater than 0.5 ug/ml, so protamine sulphate was added to fibrinogen solutions (1.5 mg/ml) at the same time as thrombin. The final protamine sulphate concentrations used were 0.1, 0.25, 0.5, 0.75 and 1.0 µg/ml.

7.2.4 Effect of heparan sulphate

According to the manufacturer, heparan sulphate (Upjohn, Kalamazoo) contained 18 U/mg of heparin activity.

It was dialysed against 2 changes of 4 litres of distilled water for 12 hours and added to fibrinogen solutions (1.5 mg/ml) to achieve final concentrations of 0.01, 0.05, 0.1, 0.5, 1 or 10 mg/ml.

7.2.5 Effect of chondroitin sulphates

Chondroitin-4-sulphate and chondroitin-6-sulphate (Sigma, U.S.A.) were freed of ionic contaminants by dialysis against 2 changes of 4 litres of distilled water

for 12 hours. Networks were formed in fibrinogen solutions (1.5 mg/ml) which contained 7, 35, 70, 350 and 700 µg/ml of either chondroitin-4-sulphate or chondroitin-6-sulphate.

7.3 RESULTS

7.3.1 Effect of glucose

Table 7.1 shows that glucose reduced permeability and hence μ_p without affecting network protein concentration when networks were formed with 1.5 U/ml thrombin. These networks were fully formed and no further increase in turbidity was recorded after 40 minutes following thrombin addition.

The μ_T and μ_p in networks formed with 0.15 U/ml thrombin were reduced in the presence of glucose (Figure 7.1) as was the network protein concentration (Table 7.2). Compaction, opacity ratio and permeability were found reduced in parallel with μ_T and μ_p (Appendix 6). These changes were apparently not influenced by divalent cations.

The decrease in mass-length ratio caused by glucose was similar to that induced by NaCl at a similar molar concentration (Figure 7.2). However, the decrease in mass-length ratio induced by glucose, but not that induced by NaCl, was associated with a marked reduction in network protein concentration.

7.3.2 Effect of protamine sulphate and heparin

Protamine sulphate increased μ_T and μ_p (Figure 7.3) but did not significantly affect the network protein concentration. Compaction, opacity ratio and permeability increased in parallel with μ_T and μ_p (Appendix 6). Divalent cations did not influence these changes.

At concentrations above 1.8 U/ml heparin significantly increased μ_T and μ_p (Figure 7.4) while below 1.8 U/ml, μ_T, but not μ_p, was reduced. These observations were confirmed in networks formed in 1.5 mg/ml fibrinogen (Figure 7.5).

The effect of heparin on opacity ratio paralleled the change in μ_T while the effect of heparin on compaction and permeability paralleled the changes in μ_p (Appendix 6). These changes were not influenced by divalent cations.

Heparin reduced the network protein concentration most effectively at about 1.8 U/ml (Figure 7.6). That is, the effect of heparin on network protein concentration paralleled the changes in μ_T but not the changes in μ_p.

Fibrinogen concentration appeared to influence the effect of protamine sulphate on network structure (Figure 7.7). 1 µg/ml protamine sulphate caused a greater increase in μ_T at lower concentrations of fibrinogen. On the

other hand, 0.5 U/ml heparin decreased mass-length ratio by a similar amount at high and low fibrinogen concentrations. 1 μg/ml protamine sulphate seemed to neutralize 0.5 u/ml heparin in its effect on the mass-length ratio.

7.3.3 Effect of heparan sulphate

Fibrin networks formed in the presence of more than 0.5 mg/ml (0.9 U/ml of heparin activity) of heparan sulphate were significantly more permeable and displayed higher opacity ratios than controls (Figure 7.8). Compaction paralleled permeability (Appendix 6). The μ_T and μ_p could not be derived because reliable measurements of the fibrin concentration in the network could not be made. This was because the protein concentration of perfusates could not be calculated on account of contamination of heparan sulphate with some material which absorbed strongly at 282 nm.

7.3.4 Effect of chondroitin sulphates

Both chondroitin-4-sulphate (Figure 7.9) and chondroitin-6-sulphate (Figure 7.10) induced similar changes in fibrin network properties. At concentrations above 7 μg/ml they significantly increased μ_T and μ_p. The chondroitin sulphates increased compaction, opacity ratio and permeability in parallel with μ_T and μ_p (Appendix 6). These changes were apparently not influenced by divalent cations.

7.4 DISCUSSION

This study demonstrates that when fibrin is formed under conditions which simulate those in plasma, the structure and properties of the fibrin network may be influenced by a variety of substances, some known to be present in the extracellular space and some in blood, naturally or under therapeutic conditions.

The studies in this Chapter support the postulate that the solubility of fibrin(ogen) strongly affects the structure of fibrin networks (Chapter 6). Protamine sulphate reduces fibrinogen solubility and increases mass-length ratio (Figure 7.3). Positively charged protamine sulphate molecules presumably precipitate fibrinogen by stoichiometrically reacting with the negatively charged fibrinopeptides (Stewart and Niewiarowski, 1969). In accordance with this suggestion, protamine sulphate increases mass-length ratio more efficiently at low concentrations of fibrinogen (Figure 7.7).

Glucose reduces mass-length ratio as well as the amount of fibrin formed by 30 minutes (Figure 7.1). The inhibition of fibrin formation by glucose was not a non-specific effect of increased osmolarity as a similar increase in osmolarity with NaCl did not reduce network protein concentration (Figure 7.2). The increased solubility of fibrin(ogen) in the presence of glucose has been attributed to steric hindrance of polymerization by

the cyclic conformation of glucose (Einarsson, 1975).

High concentrations of glycosaminoglycans, such as heparin (Figures 7.4; 7.5), heparan sulphate (Figure 7.8) and chondroitin sulphates (Figures 7.9; 7.10), presumably increase mass-length ratio as a result of a steric exclusion effect. All glycosaminoglycans occupy a large volume in solution and hence, they exert a marked steric exclusion effect on other macromolecules (Laurent, 1975; Obrink, 1975). Steric exclusion results in an effective increase in the concentration of fibrin(ogen) in solution and therefore a decrease in fibrin(ogen) solubility (Rampling et al., 1976). Hence, it is likely that steric exclusion increases mass-length ratio by decreasing fibrin(ogen) solubility.

It should be noted as discussed in the preceding Chapter that although an increase in fibrinogen concentration tends to increase mass-length ratio (Carr et al., 1977; Hantgan and Hermans, 1979; Wolfe and Waugh, 1981) this is only seen at very low fibrinogen concentrations. At physiological concentrations, the increase in the density of fibrils associated with a physical increase in fibrin concentration leads to a reduction in the extent of rotational diffusion of growing fibrils and hence a reduction in the extent of lateral aggregation. It is expected, however, that steric exclusion results in an effective increase in fibrinogen concentration without an increase in fibril density and

consequently only the tendency for an increased mass-length ratio is expressed.

Glycosaminoglycans and proteoglycans also interact non-specifically with proteins through electrostatic effects (Laurent, 1975; Obrink, 1975). Electrostatically, all glycosaminoglycans are polyanions at physiological ionic strength and pH and thus react with positively charged proteins (Laurent, 1975). Sheppard et al. (1957) proposed that heparin inhibits fibrin polymerization electrostatically. Presumably, low concentrations of heparin increase fibrin(ogen) solubility and lead to a low mass-length ratio but at higher heparin concentrations, steric exclusion effects over-ride and reduce fibrin(ogen) solubility leading to a higher mass-length ratio.

Although the effect of high and low concentrations of heparin on μ_T can be successfully explained in terms of fibrin(ogen) solubility, the influence of heparin on μ_p is unclear. Low concentrations of heparin reduced μ_T but not μ_p (Figures 7.4; 7.5). The heparin induced decrease in network protein concentration (Figure 7.6) supports the suggestion that heparin inhibits polymerization (Sheppard et al., 1957). Perhaps low concentrations of heparin also inhibit the development of the minor network (see Chapter 6) and thereby increase μ_p relative to μ_T. It should be noted that antithrombin III is not present in these systems and hence, thrombin would not be inhibited by heparin as occurs in vivo.

It is obvious from this discussion on the mechanism underlying changes in network structure that a primary cause of major changes in network structure is an alteration in fibrin(ogen) solubility. Inhibition of fibrin polymerization, that is, an increase in fibrin(ogen) solubility causes a relative decrease in mass-length ratio while a decrease in fibrin(ogen) solubility by virtue of charge or steric exclusion effects results in the opposite change in network structure. With this realization the task of identifying substances which alter network structure *in vivo* and of selecting substances for therapeutic control of fibrin network structure might become easier.

7.5 CONCLUSIONS

(1) The structure and properties of fibrin formed under conditions which simulate those in plasma are sensitive to a variety of clinically and physiologically important substances.

(2) Studies support the postulate that the solubility of fibrin(ogen) greatly influences the structure of fibrin networks.

Table 7.1 The effect of glucose on Darcy constant and network protein concentration at 1.5 U/ml thrombin.

glucose (mM)	Darcy constant x 10^{11} (cm^2)	network protein concentration (mg/dl)
Control	180.6 ± 2.9	137.3 ± 0.4
25	166.0 ± 2.0	137.9 ± 0.4
50	139.0 ± 8.5	137.4 ± 0.6
75	130.0 ± 9.6	137.3 ± 0.8
100	118.9 ± 2.1	136.5 ± 0.6

Networks were formed with 1.5 U/ml thrombin in fibrinogen solution (1.5 mg/ml) which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ using 1.5 U/ml thrombin. Networks were fully formed when perfused. Results are the mean of 3 determinations ± SD. Compare these results with those in Table 7.2 where thrombin concentration is 10 fold lower.

Table 7.2 The effect of glucose on network protein concentration at 0.15 U/ml thrombin.

glucose (mM)	network protein concentration (mg/dl)
Control	301.0 ± 2.1
10	287.2 ± 4.5
40	257.7 ± 9.4
70	214.9 ± 12.0
100	145.8 ± 10.0

Networks were formed with 0.15 U/ml thrombin in fibrinogen solutions (3.3 mg/ml) which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. Similar results were obtained when divalent cations were not present. Results are the mean of 3 determinations ± SD.

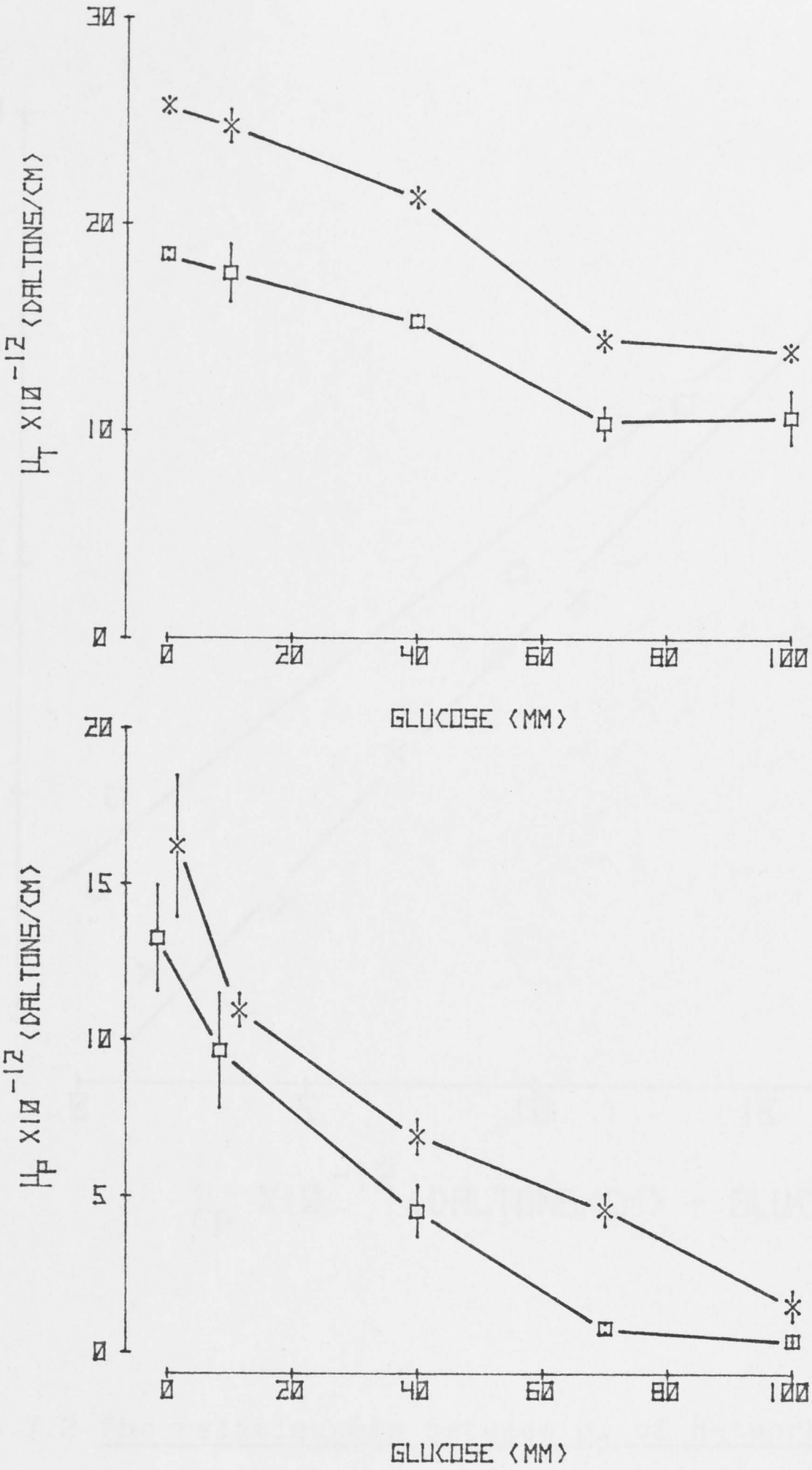


Figure 7.1 The effect of glucose on μ_T and μ_p.

Networks were formed in fibrinogen solutions (3.3 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 3 determinations ± SD.

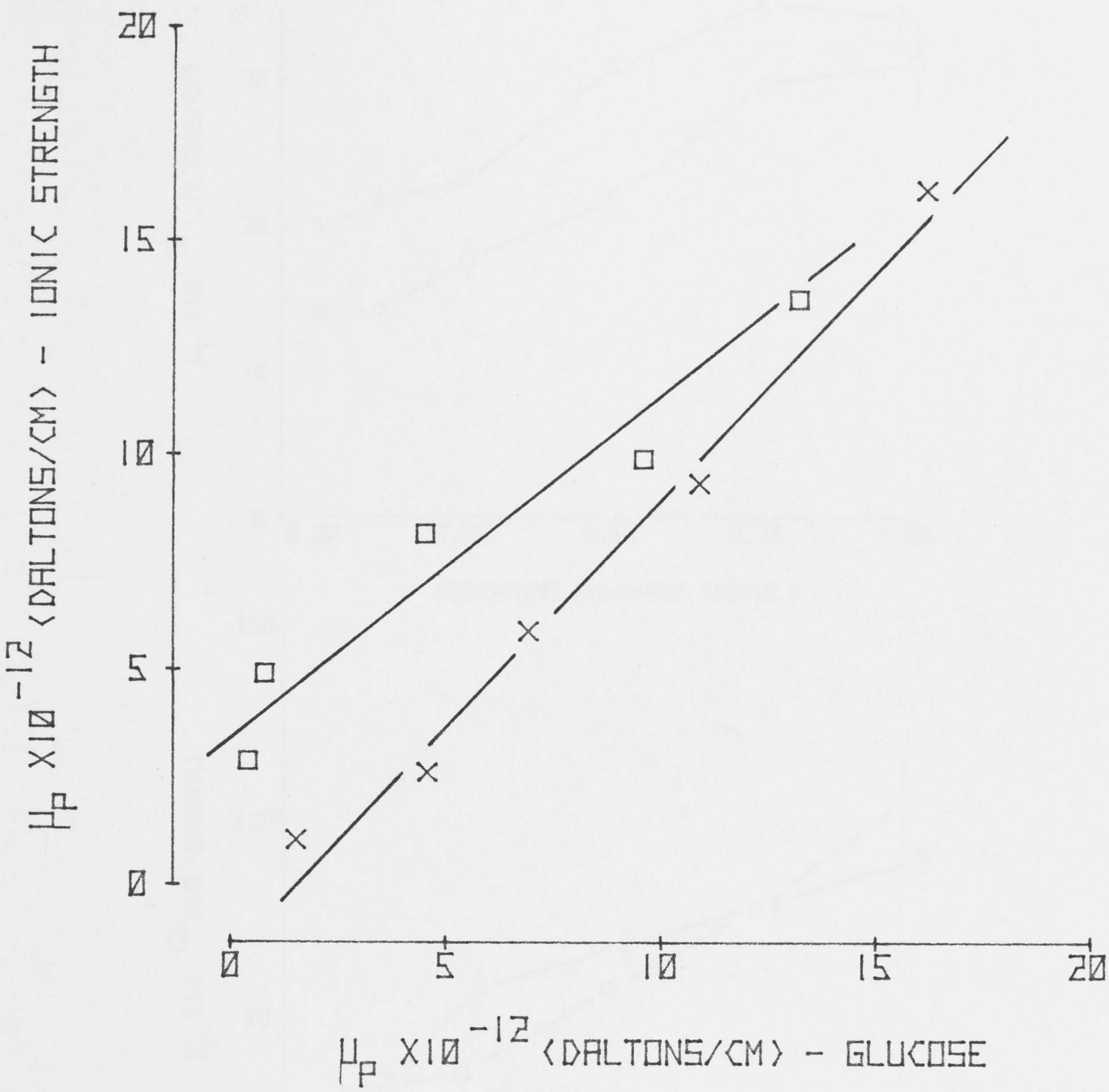
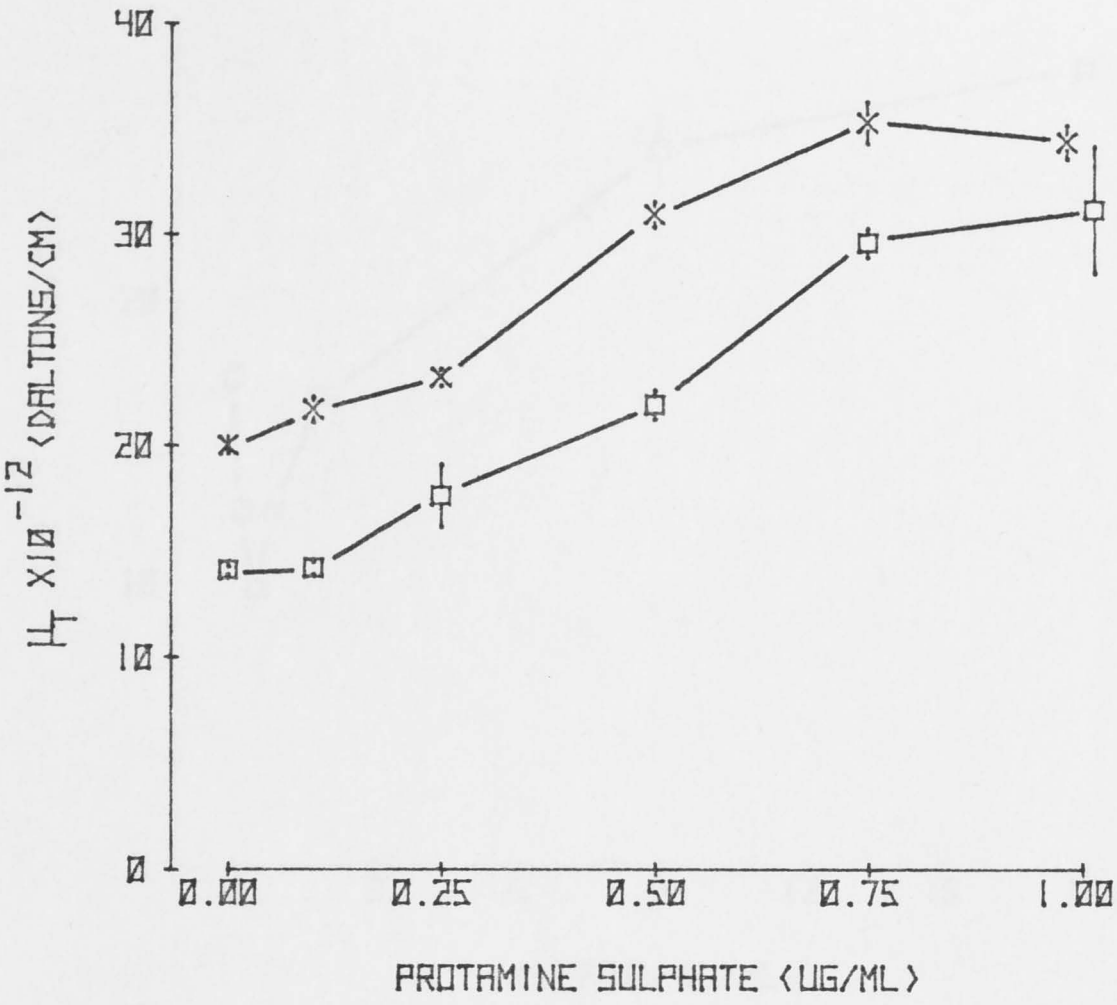


Figure 7.2 The relationship between μ_p of networks made with glucose and with NaCl of the same osmolarity

Networks were formed in fibrinogen solution (3.3 mg/ml) which either did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Mass-length ratio was altered by adding glucose or by adding NaCl of the same osmolarity. Results are the mean of 3 determinations.



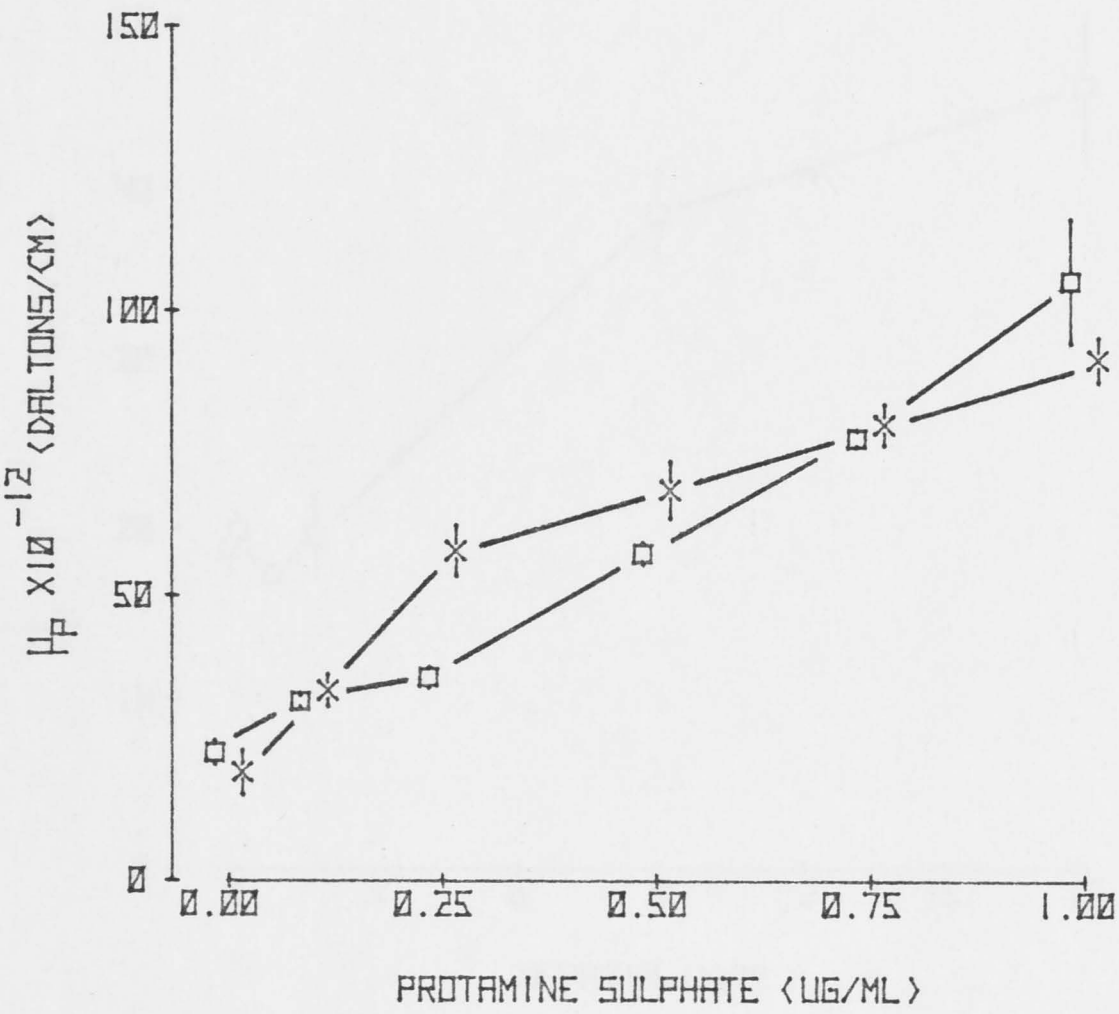


Figure 7.3 The effect of protamine sulphate on μ_T and μ_p.

Networks were formed in fibrinogen solutions (1.5 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 3 determinations ± SD.

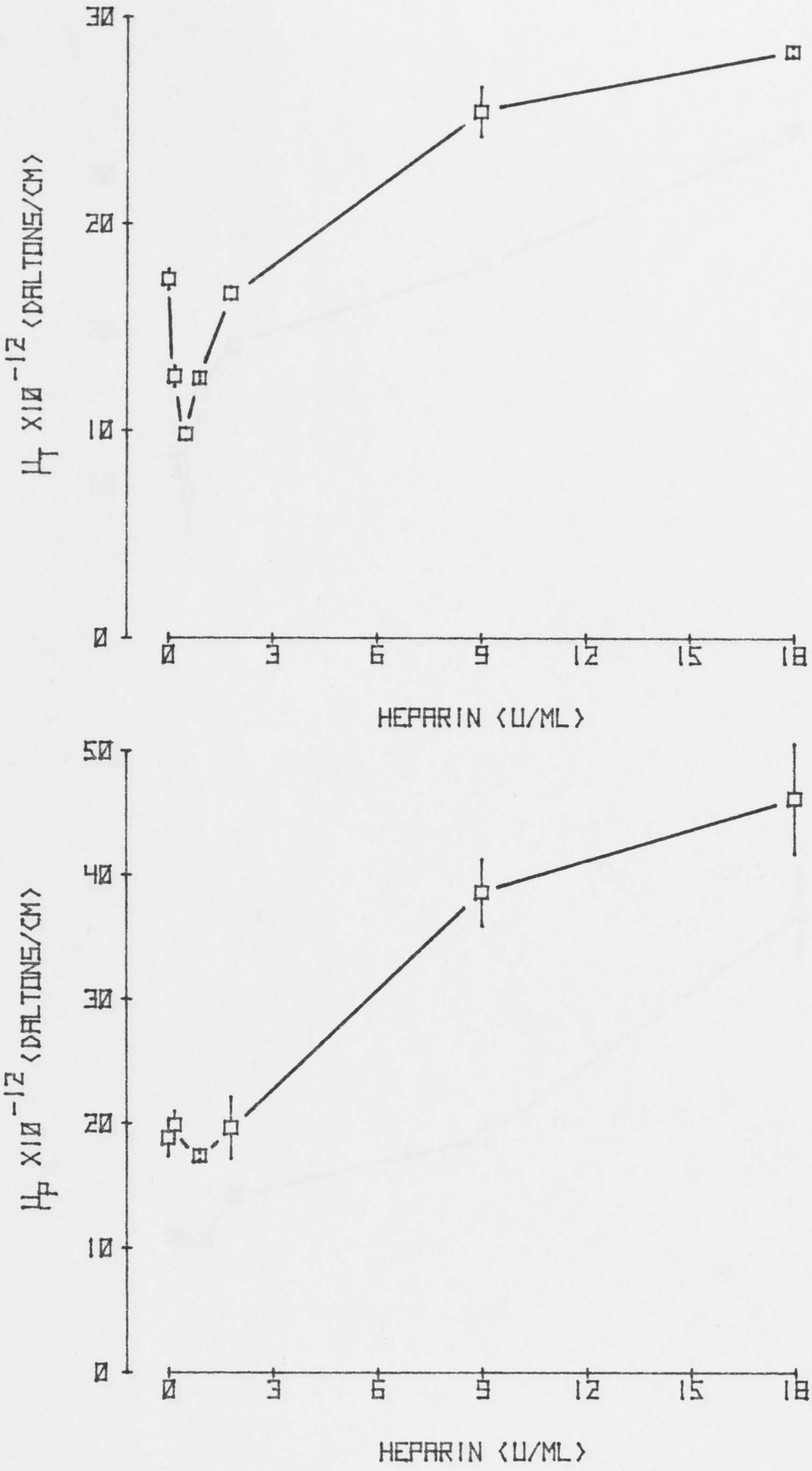
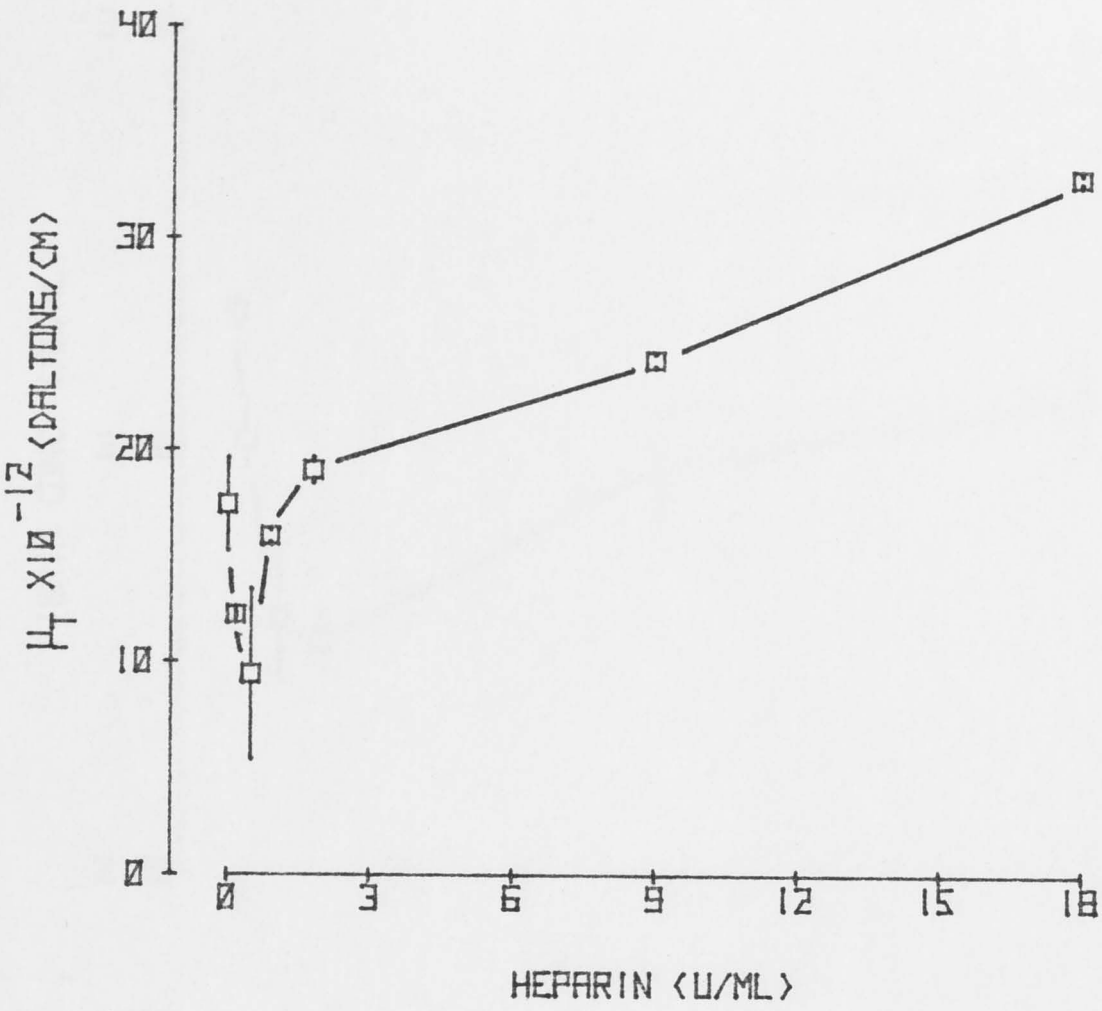


Figure 7.4 The effect of heparin on μ_T and μ_p (I).

Networks were formed in fibrinogen solutions (3.3 mg/ml) which did not contain divalent cations. Similar results were obtained in the presence of divalent cations. Results are the mean of 3 determinations $\pm$ SD.



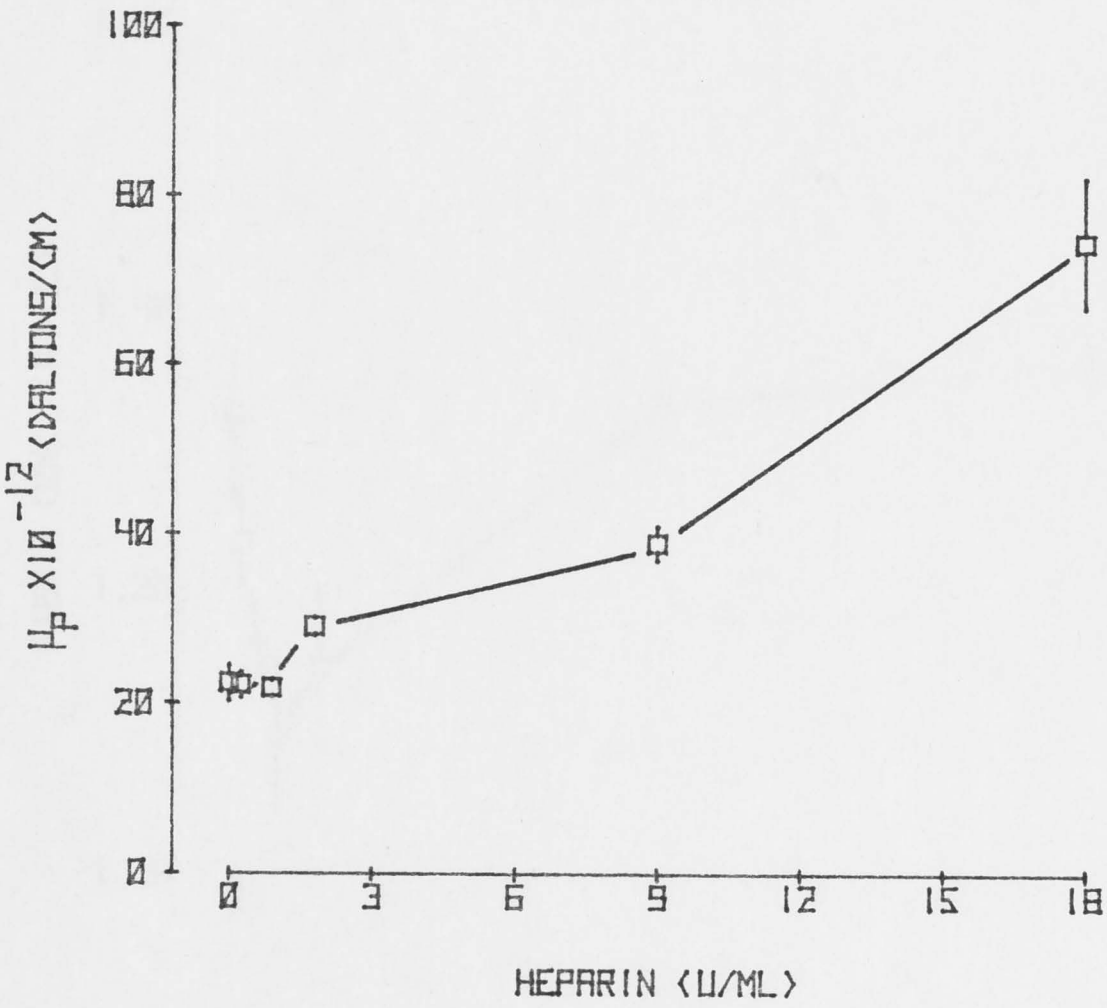
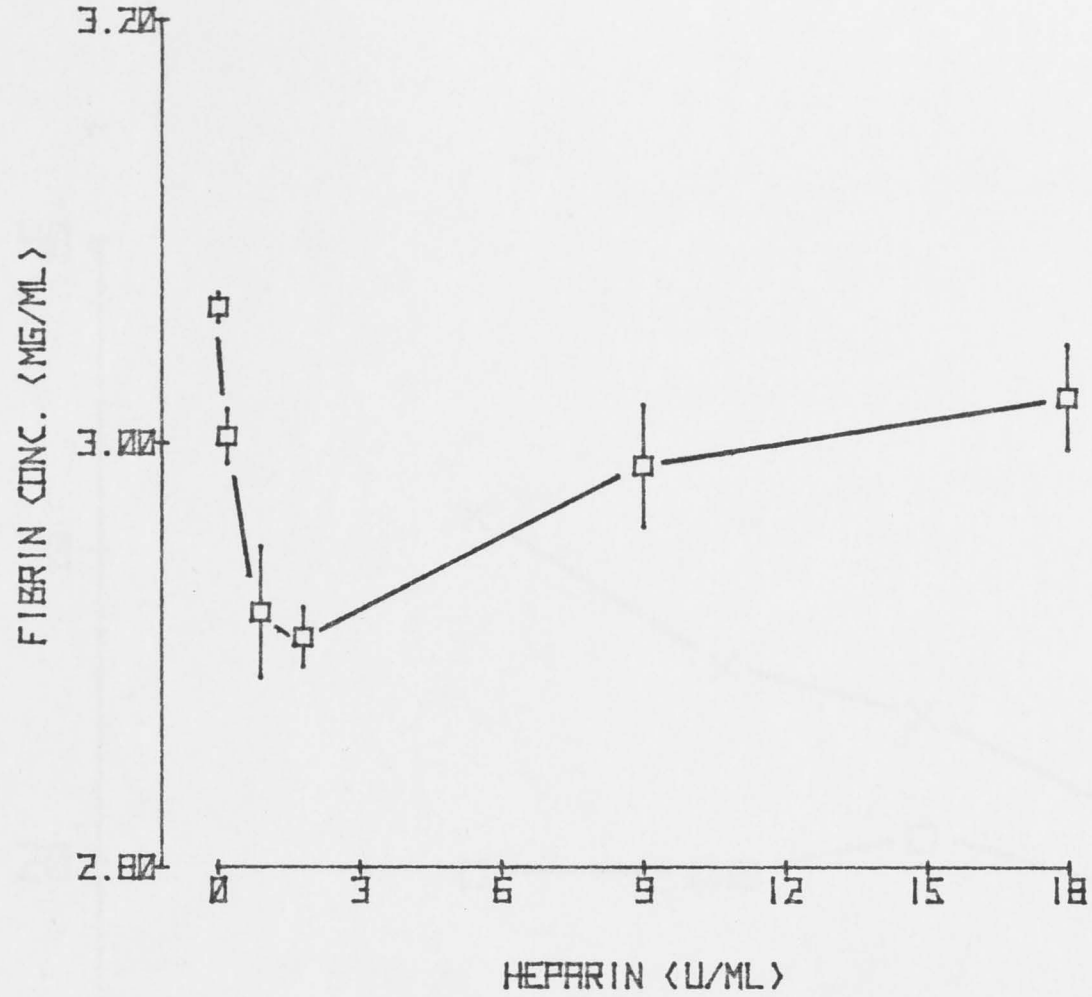


Figure 7.5 The effect of heparin on μ_T and μ_p (II).

Networks were formed in fibrinogen solutions (1.5 mg/ml) which did not contain divalent cations. Similar results were obtained with divalent cations. Results are the mean of 3 determinations $\pm$ SD.



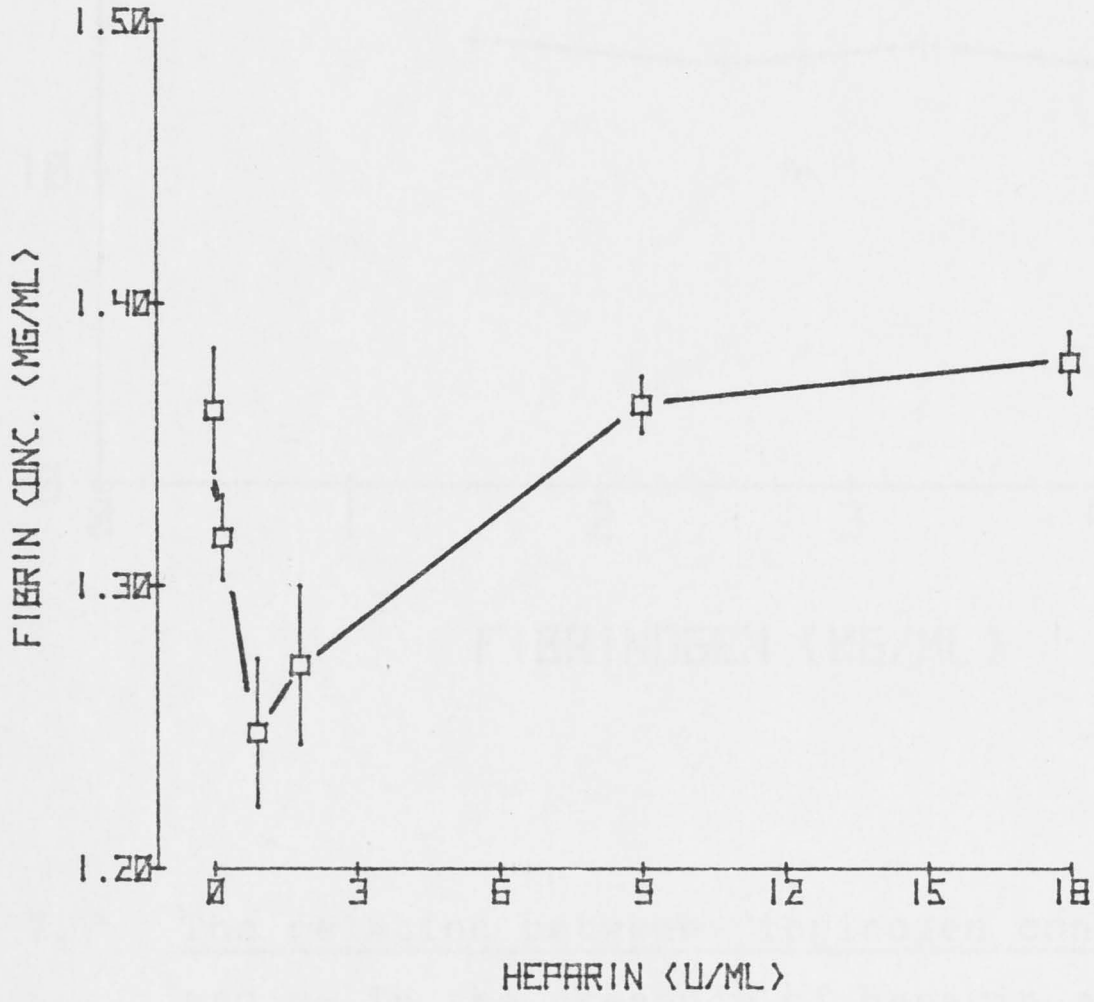


Figure 7.6 The effect of heparin on network protein concentration

Networks were formed in fibrinogen solutions (3.3 and 1.5 mg/ml) which did not contain divalent cations. Similar results were obtained when networks were formed with divalent cations. Results are the mean of 3 determinations ± SD.

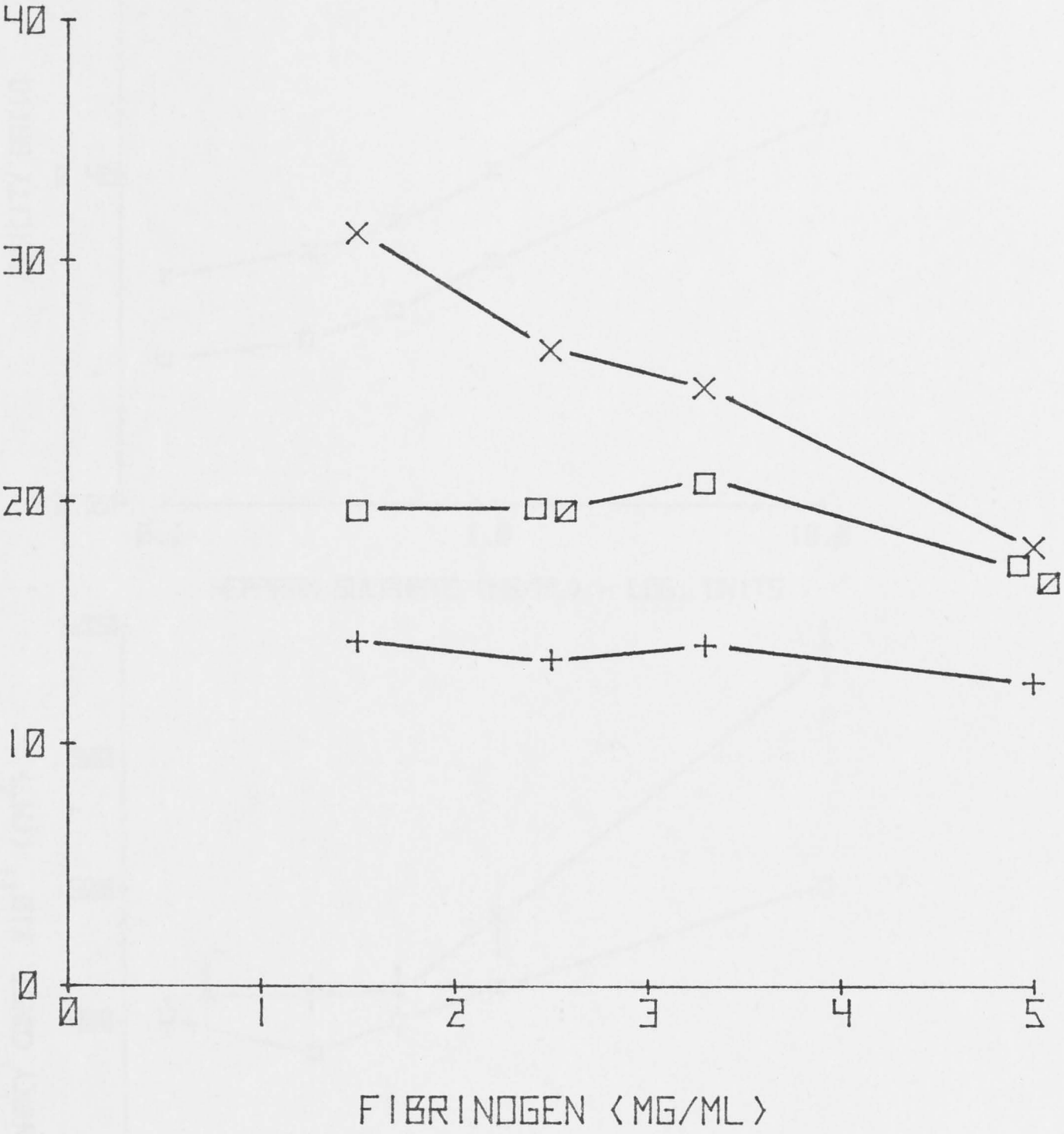


Figure 7.7 The relation between fibrinogen concentration and μ_T in the presence of heparin and protamine sulphate.

Networks were formed in fibrinogen solutions which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (□) in addition to 0.5 U/ml heparin (+) or 1 µg/ml protamine sulphate (×) or both 0.5 U/ml heparin and 1 µg/ml protamine sulphate (⊠). Results are the mean of 3 determinations.

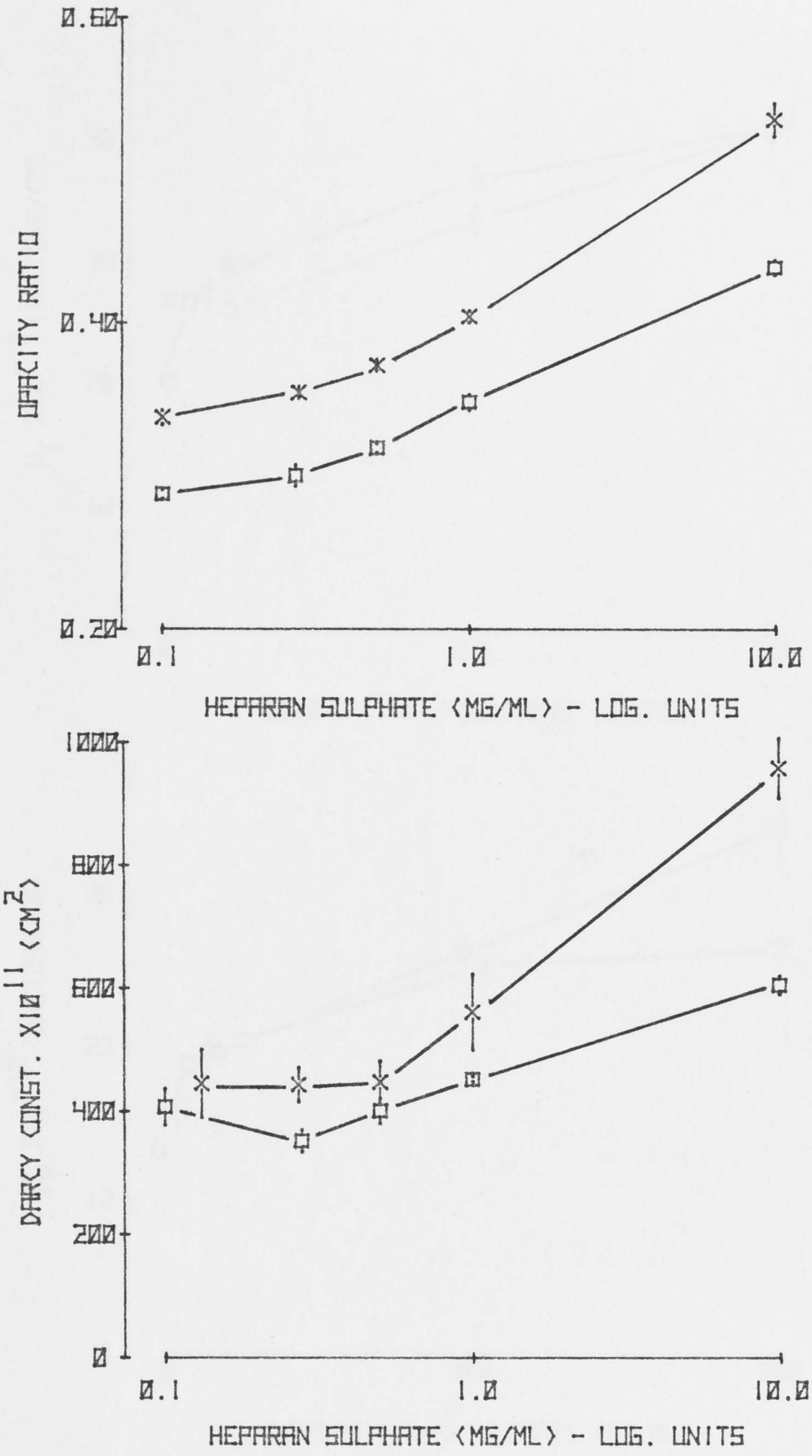


Figure 7.8 The effect of heparan sulphate on opacity ratio and permeation.

Networks were formed in fibrinogen solutions (1.5 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 determinations ± SD.

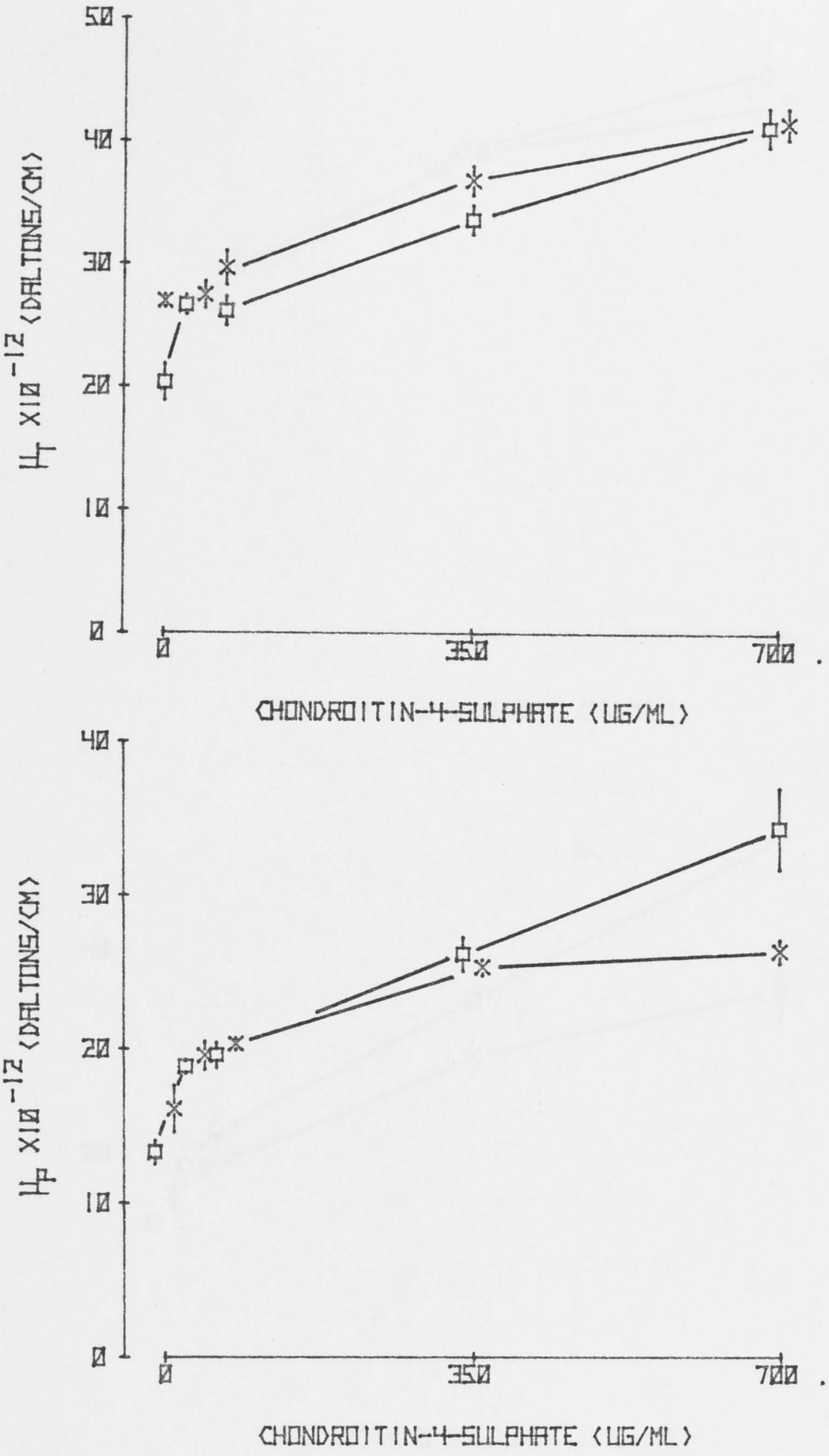
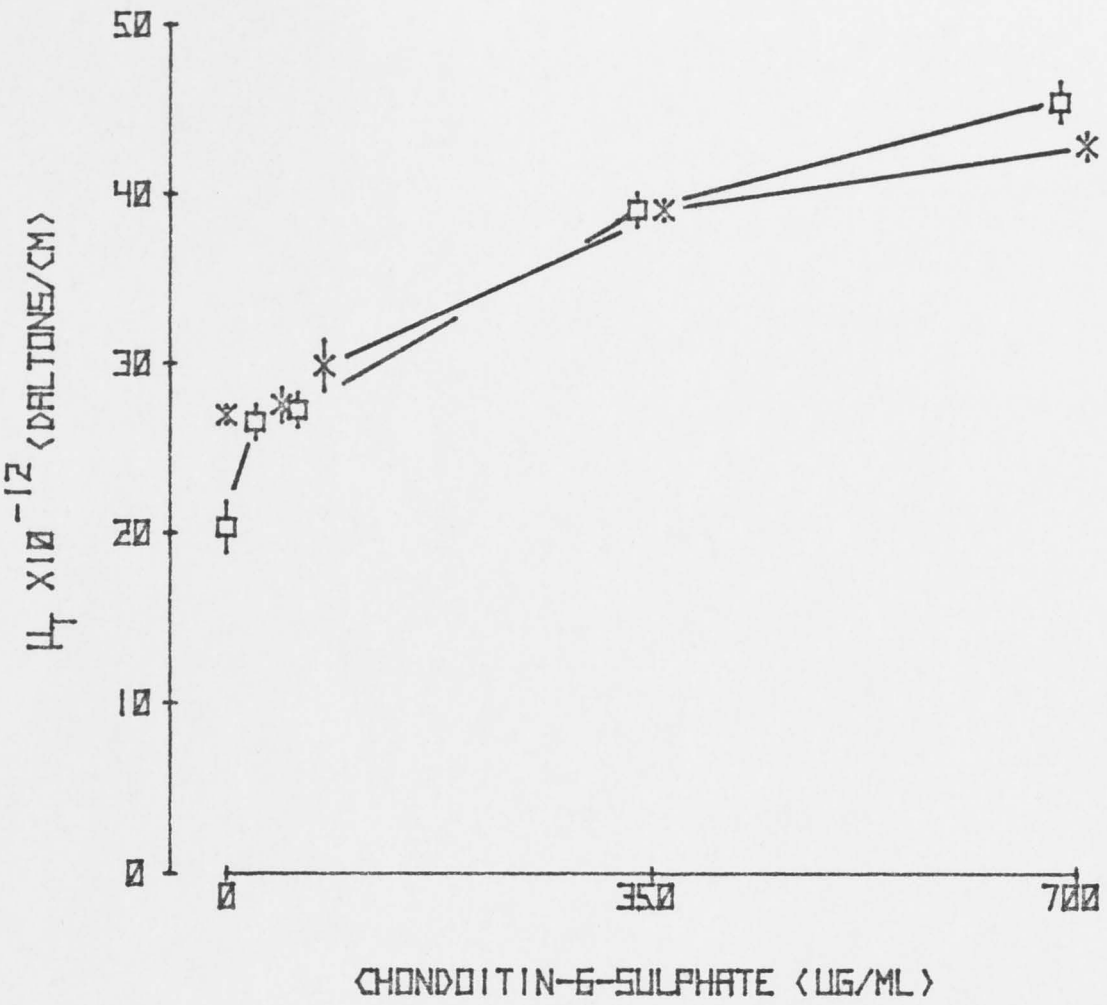


Figure 7.9 The effect of chondroitin-4-sulphate on μ_T and μ_p.

Networks were formed in fibrinogen solutions (1.5 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (×). Results are the mean of 3 determinations ± SD.



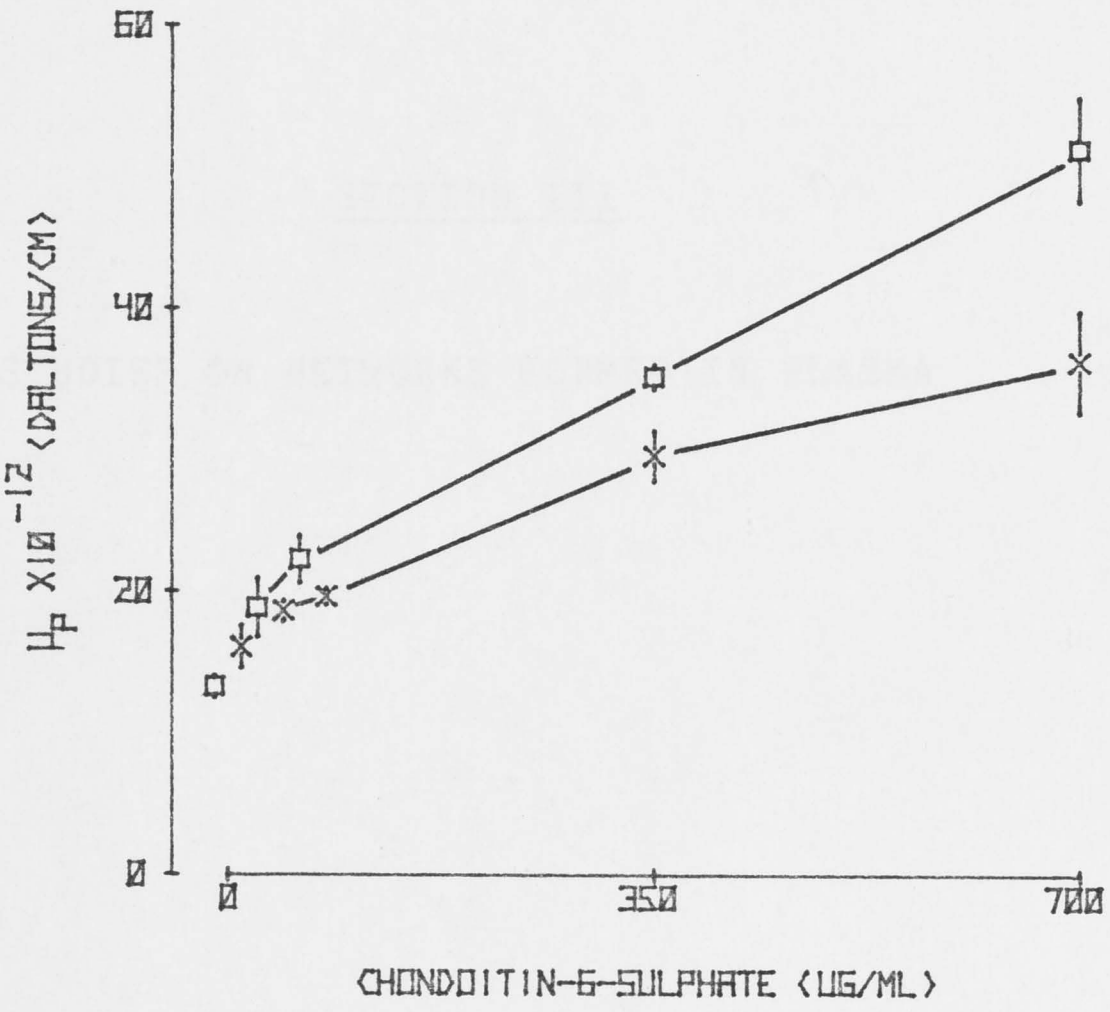


Figure 7.10 The effect of chondroitin-6-sulphate on μ_T and μ_p.

Networks were formed in fibrinogen solutions (1.5 mg/ml) which did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 determinations ± SD.

SECTION III

STUDIES ON NETWORKS FORMED IN PLASMA

CHAPTER 8

COMPARISON OF FIBRIN NETWORKS IN PLASMA AND FIBRINOGEN SOLUTION AND THE EFFECT OF ANTICOAGULANTS AND HANDLING PROCEDURES

8.1 INTRODUCTION

Fibrin network formed in plasma differs from that formed in purified fibrinogen solutions in two fundamental ways. During network development in plasma in the absence of anticoagulants, the thrombin concentration increases explosively (Liu et al., 1979). In anticoagulated plasma and in purified fibrinogen solutions, however, the thrombin concentration does not increase during network development. Networks formed in plasma also differ from those formed in purified solutions in that many plasma components such as immunoglobulins (Regeoczi, 1968), fibronectin and α_2-antiplasmin (Mosher, 1980) are incorporated into the network matrix. It was not known whether basic differences in network development and composition affect the structure and properties of fibrin networks and if so, whether these differences are affected by the anticoagulant used or by the procedure used to prepare the plasma.

Studies described in this Chapter compare networks formed in plasma prepared under different conditions with networks formed in serum reconstituted with fibrinogen and with networks formed in purified fibrinogen solutions.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of plasma clots

8.2.1.1 Blood donors

Donors had fasted overnight and had not taken any drugs known to affect platelet function or blood coagulation for 10 days. All donors were healthy and had normal full blood and differential counts as measured with a Technicon Hemalog. Blood was drawn by atraumatic venepuncture using 21 gauge needle, 20 ml plastic syringes and mild compression with tourniquet and collected into different anticoagulants as described in 8.2.2, 8.2.3 and 8.2.4.

8.2.1.2 Plasma

Unless otherwise stated, blood was centrifuged for 15 minutes at 2500 g in a MSE multex bench centrifuge at room temperature. In some studies, a MSE Minstral 6L centrifuge was used for centrifuging at 2°C. The resulting platelet-poor plasma (PPP), was removed with a siliconized pasteur pipette. Platelet-free plasma (PFP) was prepared by centifuging PPP for 10 minutes at 10,000 g in a MSE high speed 25 at room temperature.

8.2.1.3 pH measurements

The pH of blood was measured with a Radiometer ABL-1 Acid-Base Laboratory. The pH of the anticoagulants was measured with a PHM 62 standard pH Meter (Radiometer, Copenhagen).

8.2.1.4 Buffered citrate

A mixture of 0.2M trisodium citrate and 0.2M acid citrate of pH 6.80 (hereafter termed buffered citrate) was sometimes used as an anticoagulant. Preliminary studies showed that blood collected into 0.2M trisodium citrate in ratio 19:1 had a pH of about 7.7, while blood collected in the same ratio into 0.2M buffered citrate had a pH of 7.404 $\pm$ 0.015 (n=6).

8.2.1.5 Fibrin networks in plasma

Fibrin networks were developed in plasma by clotting 960 ul of plasma with 40 ul of 3.75 U/ml thrombin dissolved in either 0.6M $CaCl_2$ (thrombin /$CaCl_2$) or 0.895% saline. Where required, 10 µl (approximately 10^7 counts/min) of ^{125}I-labelled fibrinogen was added to 950 µl of plasma prior to clotting. Radioactivity counts were made (Chapter 2).

8.2.1.6 Perfusion of networks in plasma

In preliminary studies, networks formed in plasma with 0.15 U/ml thrombin sometimes collapsed under 15 cm pressure head of water. It was therefore decided to perfuse all plasma clots at a pressure head of 10 cm of water and to precoat the internal surfaces of the perfusion tubes (Carr et al., 1977) with a film of purified fibrin containing Ca^{++} and Mg^{++} (Chapter 2) so as to obtain

good adherence of plasma clots to perfusion tubes. The fibrin film was left for 1 hour before plasma was added and clotted. Permeability was measured as otherwise described in Chapter 2.

8.2.1.7 Networks in fibrinogen solution

Unless otherwise stated, fibrin networks were formed with 0.15 U/ml thrombin in fibrinogen solution (3.3 mg/ml) which either did not contain divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (Chapter 2). Networks were examined using the methods described in Chapter 2.

8.2.1.8 Electron microscopy

Blood was collected into 0.2M buffered citrate in the ratio of 19:1 and PPP prepared. Networks were formed using thrombin (1.5 U/ml) and specimens prepared for transmission and scanning electron microscopy as described in Chapter 2. A similar procedure was followed for forming networks in fibrinogen solution containing divalent cations.

8.2.2 Networks in serum

9.5 ml of blood was collected into tubes which contained 60 µl ^{125}I-fibrinogen and 0.5 ml of 0.2 M buffered citrate. Some of the anticoagulated blood was processed to yield PFP, and the remainder was clotted with

thrombin/$CaCl_2$. Some of the PFP was also clotted with thrombin/$CaCl_2$. The final thrombin concentration was 0.15 U/ml. The serum from coagulated blood and plasma specimens was removed 2 hours later. The thrombin concentration in the serum was determined using a thrombin clotting time standard curve prepared with stock PFP (collected from 10 blood donors, pooled and stored at -77°C), thrombin standards and a fibrometer (BBL,USA). Further thrombin was added to the serum to bring the thrombin concentration to 1 U/ml. These sera were then reconstituted with fibrinogen which was adjusted to the same level as in the plasma. PFP was also clotted with thrombin/$CaCl_2$ so that the final concentration was 1 U/ml. The fibrinogen concentration was determined using the heat precipitate determination of Goodwin (1965). All clots were left to develop fully for 1 hour before μ_T and μ_p were determined.

8.2.3 Effect of anticoagulant and recalcification

9.5 ml blood was collected into chilled tubes kept on ice. These tubes either did not contain any solution or contained 0.5 ml of 0.2 M buffered citrate or 0.5 ml of 1.2 M NaCl. The tubes were immediately centrifuged at 2°C. PPP was removed and warmed to room temperature in a water bath. The PPP was then clotted with thrombin/$CaCl_2$ or thrombin alone. The opacity ratio and compaction were measured 30 minutes later (Chapter 2).

8.2.3.1 Effect of citrate

A mixture of acid citrate and trisodium citrate at pH 7.4 was used to give final citrate concentrations of 2.5, 5.0, 7.5, 10.0 or 12.9 mM. The ionic strength of these solutions was maintained at 0.153.

8.2.4 Effect of anticoagulants, temperature and platelet release products

9.5 ml of blood was collected into 0.5 ml of 0.2M buffered citrate or CTP (see 9.2.4.1 for final concentrations of components). In addition, 9.0 ml of blood was mixed with 1 ml of 3.8% trisodium citrate. These anticoagulated blood samples were either immediately chilled on crushed ice and centrifuged at $2^{o}C$ to yield PPP which was then warmed to room temperature in a water bath, or the blood was processed entirely at room temperature. The PPP was clotted with either thrombin/$CaCl_2$ or thrombin alone. The opacity ratio and compaction were measured 30 minutes later.

8.2.4.1 Effect of citrate-theophylline-PGE_1 solution

Fibrinogen solutions with 10 mM buffered citrate (pH 7.4) was used as the control. To this was added (final concentration) : either 10 mM theophylline (Sigma, USA) or 0.001% ethanol or 0.1 µg Prostaglandin E_1 (Sigma, USA)

dissolved in 0.001% ethanol or both Prostaglandin E_1 and theophylline.

8.2.4.2 Effect of citrate-phosphate-dextrose solution

CPD from unused blood packs (Tuta, Sydney) was added to fibrinogen solution or blood in a ratio of 1:6.8. The pH was recorded and networks formed. In separate experiments, the pH of these solutions was readjusted to 7.4 with HCl before clotting.

8.2.4.3 Effect of platelet release products

PF_4, βTG and γTG were gifts from Dr. F.J. Morgan (St. Vincent's School of Medical Research, St. Vincent's Hospital, Melbourne). These were dissolved in distilled water and added to fibrinogen solutions to achieve final concentrations of 5, 10, 50 and 100 μg/ml.

8.3 RESULTS

8.3.1 Networks formed in plasma and purified solutions

Table 8.1 shows that networks formed in plasma compact more easily than purified fibrin networks. Although both μ_T and μ_p of plasma networks are greater than those established for purified fibrin networks formed under patho-physiological conditions, the μ_p of plasma networks

is much larger than would be expected from the ratio of μ_p to μ_T found in purified fibrin networks.

Under transmission electron microscopy, fibrin networks formed in plasma appear identical to networks formed in purified fibrinogen solutions (Figure 8.1). Under scanning electron microscopy, however, fibrin networks formed in plasma were different to those formed from purified fibrinogen. The fibrin fibres of plasma clots seemed compacted together as if deformed during the freeze-drying process (Figure 8.2).

8.3.2 Networks formed in serum

Figure 8.3 shows that the μ_p of networks in plasma is substantially higher than that of networks in reconstituted sera. The μ_T of networks in plasma, however, is significantly lower than that in reconstituted sera. The ratio of μ_T to μ_p is much lower in networks in plasma than in reconstituted sera.

The μ_T of clots formed in PFP serum is not significantly different from that of clots formed in serum from anticoagulated blood (Figure 8.3). The μ_p of the former, however, is significantly lower ($p(F) < 0.0171$) than that of the latter.

8.3.3 Effect of anticoagulant and recalcification

The effect of anticoagulants and recalcification on networks in plasma is shown in Figure 8.4. The opacity ratio and compaction of networks in anticoagulated PPP with thrombin/$CaCl_2$ are not significantly different from those in native PPP. When anticoagulated plasma was clotted without recalcifying the opacity ratio was reduced and compaction increased relative to the values in native PPP. NaCl of the same ionic strength as 0.01M citrate decreased both opacity ratio and compaction.

Networks made in native PPP or in anticoagulated PPP with thrombin/$CaCl_2$ showed significant retraction and sometimes an opacity ratio greater than 1.000. This was never observed in networks in PFP or in PPP with thrombin alone.

When PFP (made with 0.2M buffered citrate and containing ^{125}I-fibrinogen) was clotted with thrombin/$CaCl_2$, 92.4 $\pm$ 3.1% of the ^{125}I-fibrinogen was converted into network. However when thrombin alone was used for clotting only, 42.5 $\pm$ 4.1% of the label was incorporated into the network by 30 minutes (n=10).

Citrate alone significantly increased μ_T and μ_p in purified fibrin at constant ionic strength and pH (Figure 8.5). Citrate increased compaction, opacity ratio and permeability in parallel with μ_T and μ_p. These changes

were apparently not influenced by divalent cations.

8.3.4 Effect of anticoagulants, temperature and platelet release products

Opacity ratio and compaction of networks formed in PPP (Figure 8.4) is greater than the corresponding value in PFP (Figure 8.6).

The temperature at which PPP is prepared and the presence of theophylline or PGE_1 does not significantly affect opacity ratio or compaction (Figure 8.6).

Theophylline and PGE_1, do not significantly affect the opacity ratio or permeability of purified fibrin networks (Figure 8.7). A very low concentration of ethanol increases opacity ratio and permeability slightly.

The opacity ratio and compaction in networks formed using thrombin/$CaCl_2$ in PFP containing 3.8% trisodium citrate were significantly higher ($p(F)<0.0008$ and 0.0026 (4^oC); $p(F)<0.0261$ and 0.0002 (22^oC), respectively) than those in networks formed in PFP containing buffered citrate (Figure 8.5). However, these values proved to be linearly correlated ($p(F)<0.0152$ and 0.0001 (4^oC); $p(F)<0.0001$ and 0.0001 (22^oC), respectively). Further, the opacity ratio and compaction in networks formed using thrombin alone in PFP containing 3.8% trisodium citrate were linearly correlated ($p(F)<0.028$ and 0.0003 (4^oC);

p(F)<0.0031 and 0.0071 (22°C), respectively) with those in networks formed using thrombin/$CaCl_2$ in PFP containing buffered citrate.

Three platelet proteins known to be released during coagulation were found not to significantly influence the mass-length ratio in purified fibrin network (Figure 8.8).

Blood collected in CPD had a pH of near 6.0. Networks formed very slowly in plasma prepared from CPD blood, even at pH 7.4. These networks could not withstand pressure heads of 5 cm during permeation and collapsed completely when examined for compaction.

As Table 8.2 shows, CPD also dramatically affected networks formed in purified fibrinogen. The pH of the buffered fibrinogen solution with CPD was about 6.5. These networks were not fully formed at 30 minutes as indicated by the large perfusate protein concentration. Consequently, these networks exhibited large permeability and compaction relative to the control. On the other hand, when the pH of CPD-fibrinogen solutions was readjusted to that of the control, network permeability and compaction were much lower than those in the control. They were so low, in fact, that perfusate could not be collected nor compaction measured. Hence, mass-length ratio could not be calculated.

8.4 DISCUSSION

Although networks formed in plasma are similar to those formed in purified fibrinogen solution in terms of their appearance under the transmission electron microscope (Figure 8.1) they differ in their ability to resist deformation during freeze drying (Figure 8.2) and centrifugation (Table 8.1). Relative to purified fibrin networks, networks formed in plasma are considerably more permeable than their μ_T would indicate (Table 8.1). It is thus obvious that networks formed in plasma exhibit different mechanical properties to purified fibrin networks.

The reason underlying the differences in network characteristics in plasma and in fibrinogen solution is not known. Networks formed in serum do not display the relatively great permeability which seems such a characteristic feature of networks in plasma (Figure 8.3) The enhanced permeability does not arise from deformation under perfusion conditions (Appendix 3). It seems possible that some plasma component is responsible for perfusion characteristics of networks developed in it. Furthermore, because such an enhanced permeability does not feature prominently in networks made in reconstituted sera, it may be that the responsible plasma component is in some way consumed during network development.

It is known that some plasma components which are

incorporated into networks may alter their tensile properties and hence the ease with which networks deform. For example, fibronectin, which comprises about 4% of the mass of the network formed in plasma (Mosher, 1980), considerably increases the elastic modulus of fibrin, presumably by binding fibres together (Kamykoski et al., 1981).

The relative absence of the minor network in plasma networks may also explain their relatively great permeability and deformability. Networks formed in native plasma characteristically contain no fibres of less than 100nm diameter (Koppel, 1962). Perhaps, unlike networks formed in serum and fibrinogen solution, the minor network does not develop where thrombin concentration increases explosively in native (Shuman and Majerus, 1976) and recalcified anticoagulated plasma (Liu et al., 1979). In other words, the amount of minor network formed when networks are developed with a fixed thrombin concentration may be greater than where the thrombin concentration increases sharply.

Unlike networks formed in anticoagulated plasma using thrombin alone (0.15 U/ml), the progressive increase in thrombin concentration in plasma clotted using thrombin/$CaCl_2$ (Liu et al., 1979) results in almost complete conversion of fibrinogen into fibrin network. The lower fibrin concentration in networks formed in anticoagulated plasma using thrombin alone accounts for

their relatively low opacity ratio and high compaction (Figures 8.4 and 8.6).

Differences in thrombin concentration resulting from the generation of additional thrombin in recalcified plasma can explain the relatively small differences in μ_T of networks formed in plasma and reconstituted serum (Figure 8.3). Thus, although platelets contain substances which reduce mass-length ratio (Dhall et al., 1982), it seems unlikely that cellular elements of blood release components which substantially increase mass-length ratio. Greater than serum concentrations (Bolton et al., 1976, Prowse et al., 1980) of platelet release products do not significantly affect mass-length ratio (Figure 8.8), and neither does collecting the blood under conditions known to inhibit release of platelet products viz collection of blood into CTP on ice (Jakubowski, 1980), (Figure 8.6). It thus seems likely that the very thick fibrin fibres found associated with platelets in thrombi (Gottlub, 1975; Hattori et al., 1978; Hisano, 1978) and haemostatic plugs (Sixma and Wester, 1977) are not caused by enhanced lateral polymerization due to platelet release products, but rather they may be simply thinner fibrin fibres which have been drawn together during platelet mediated clot retraction (James et al., 1960; 1962; Szalontai, 1968).

It is apparent that the structure of networks formed in plasma is sensitive to the anticoagulant used. Anticoagulant concentration (Figure 8.5), pH (Table 8.2)

and ionic strength (Figure 8.4), all influence the network characteristics. But to a large extent, the effect of anticoagulant on the fibrin content of networks formed in plasma appears to be neutralized by recalcification (Figure 8.4).

This might suggest that the anticoagulant in transfused blood will not affect fibrin network structure and properties provided enough Ca^{++} is available in the circulation to neutralize the citrate load. This may not be the case where large volumes of blood are administered clinically. In such cases, circulating high concentrations of citrate, glucose and phosphate may affect the development of fibrin network structure in vivo. This is an area which will require full examination in the future.

8.5 CONCLUSIONS

(1) The networks formed in plasma are more permeable and deform more readily than networks formed in purified fibrinogen solutions.

(2) The networks formed in plasma are sensitive to anticoagulants, ionic strength and pH but, to a large extent, the effect of anticoagulants is neutralized by recalcification.

(3) The cellular elements of blood do not release stable serum soluble factors which increase the mass-length ratio.

Table 8.1 Comparison of μ_T, μ_p and compaction of networks formed in plasma and in purified fibrinogen solutions.

	NETWORKS FORMED IN	
	PLASMA	PURIFIED FIBRINOGEN SOLUTIONS
$\mu_T \times 10^{12}$ (daltons/cm)	24.6 ± 3.1	15.1 ± 4.2
$\mu_p \times 10^{12}$ (daltons/cm)	83.4 ± 34.9	20.1 ± 8.8
Compaction (%)	67.8 ± 5.4	10.0 ± 4.6

Networks were formed either in PFP using buffered citrate and thrombin/$CaCl_2$ (results expressed as the mean of at least 5 observations ± SD) or in purified fibrinogen solution containing 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ where ionic strength, pH, temperature thrombin and fibrinogen concentrations were varied within physiological ranges (results from Chapter 6 expressed as the mean of at least 26 observations ± SD).

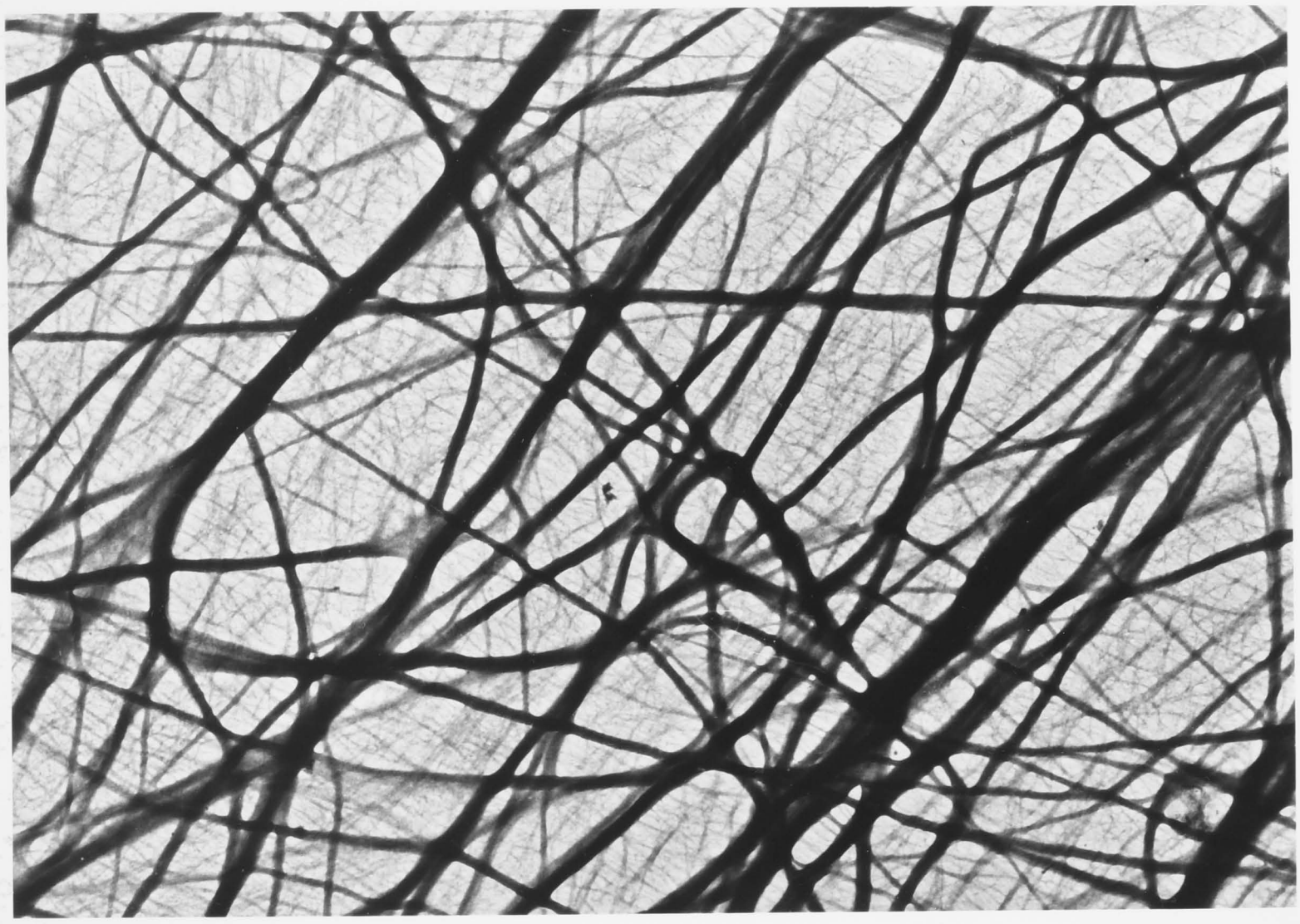
Table 8.2 The effect of CPD at different pHs on opacity ratio, permeability, compaction and network protein concentration

	Opacity ratio	Darcy constant $x10^{11}$ cm^2	Compaction (%)	Fibrin conc. (mg/ml)
Control	0.78 ± 0.04	68.1 ± 9.5	9.2 ± 0.7	3.02 ± 0.03
CPD-pH 6.5	0.69 ± 0.05	1046 ± 48	32.5 ± 1.0	1.19 ± 0.02
CPD-pH 7.4	0.31 ± 0.01	3.4 ± 0.1	NA	NA

Networks were formed in fibrinogen solution (3.3 mg/ml) of different pHs which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. Similar results were obtained in the absence of divalent cations. Results are the mean of 3 observations ± SD.

Figure 8.1 Transmission electron micrographs of fibrin networks formed in plasma (above) and in purified fibrinogen solution

The magnification of these micrographs is the same.



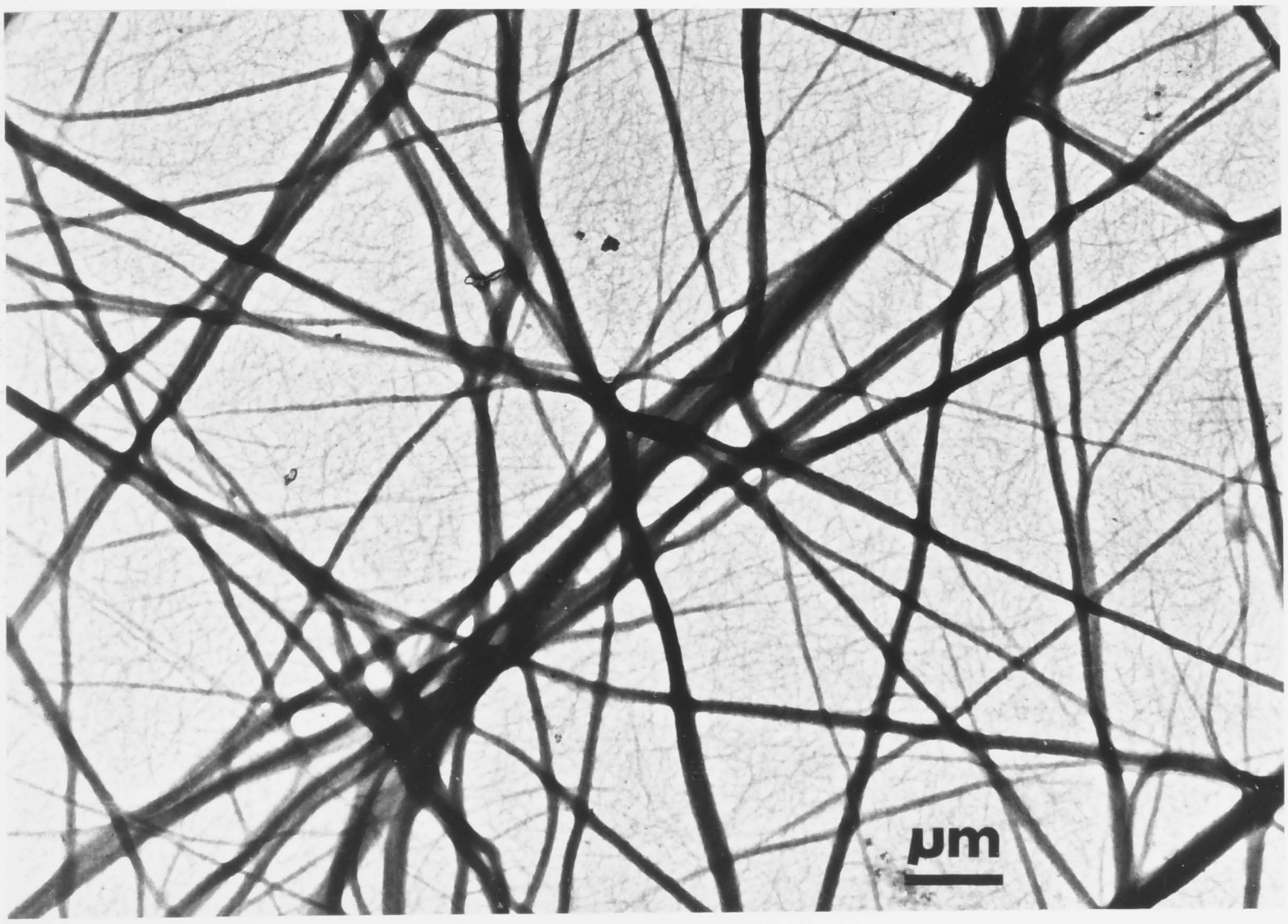
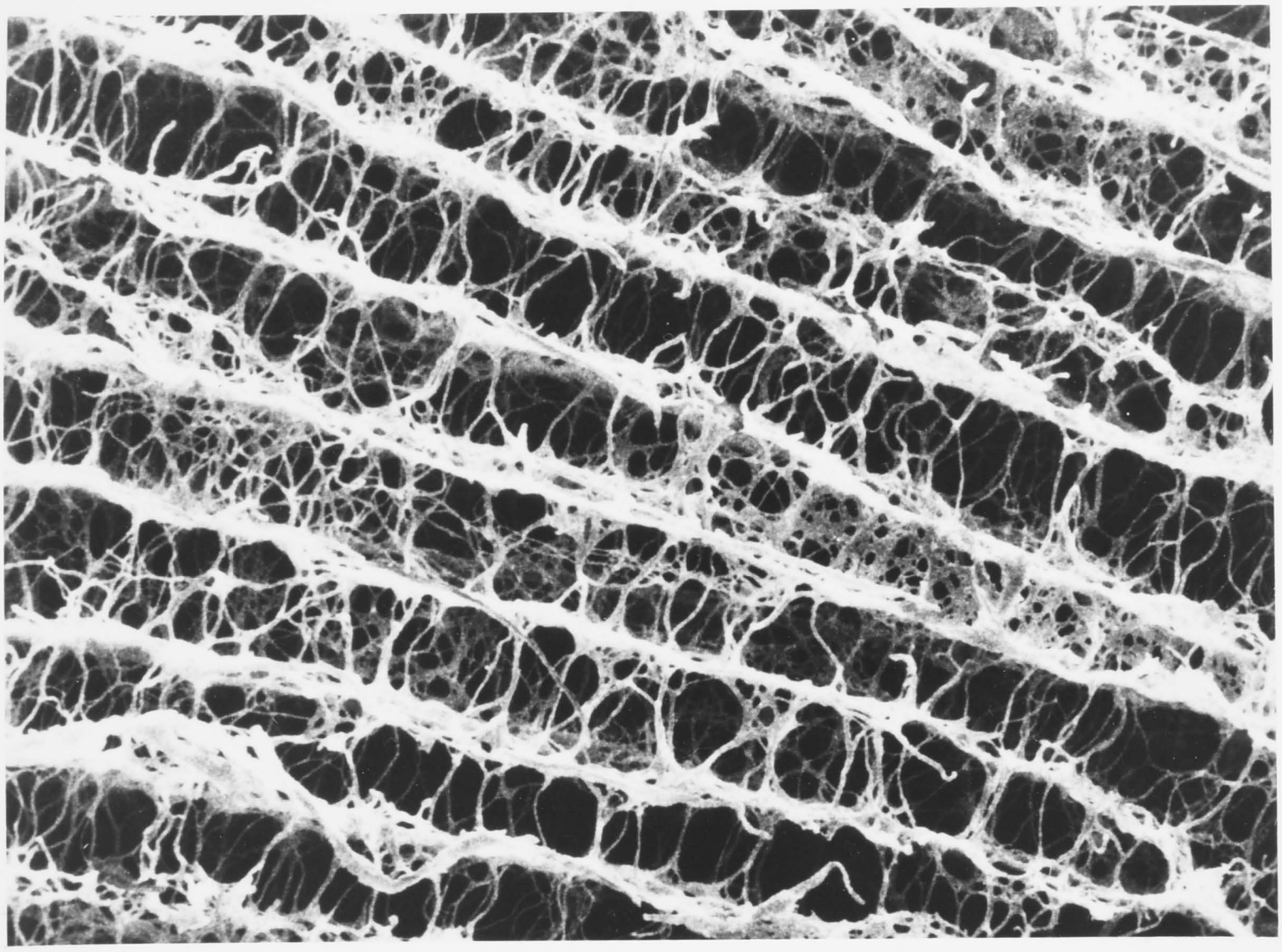
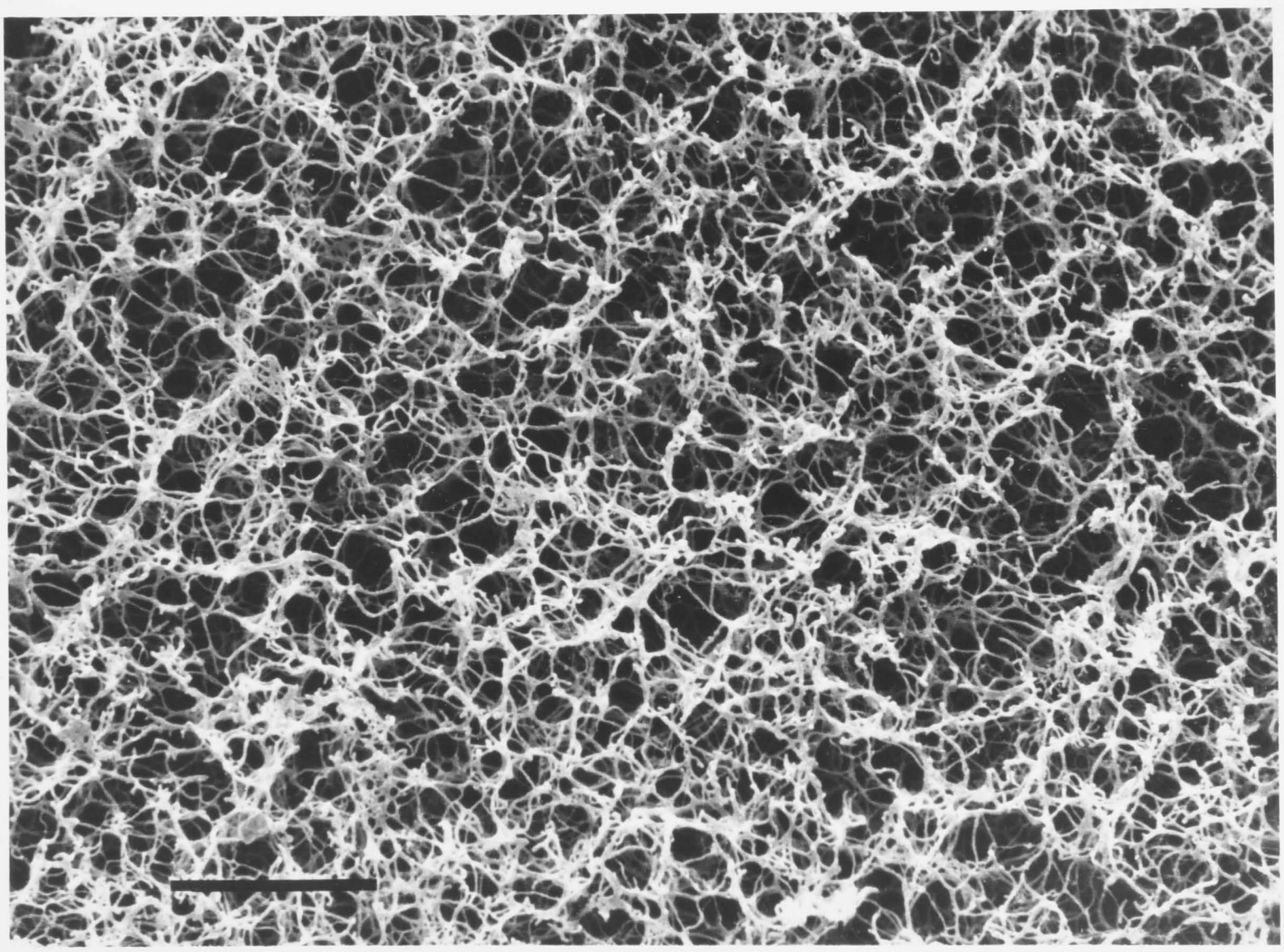

µm

Figure 8.2 Scanning electron micrographs of fibrin network formed in plasma (above) and in purified fibrinogen solution

Scale is 20μm and the magnification of these micrographs are the same.





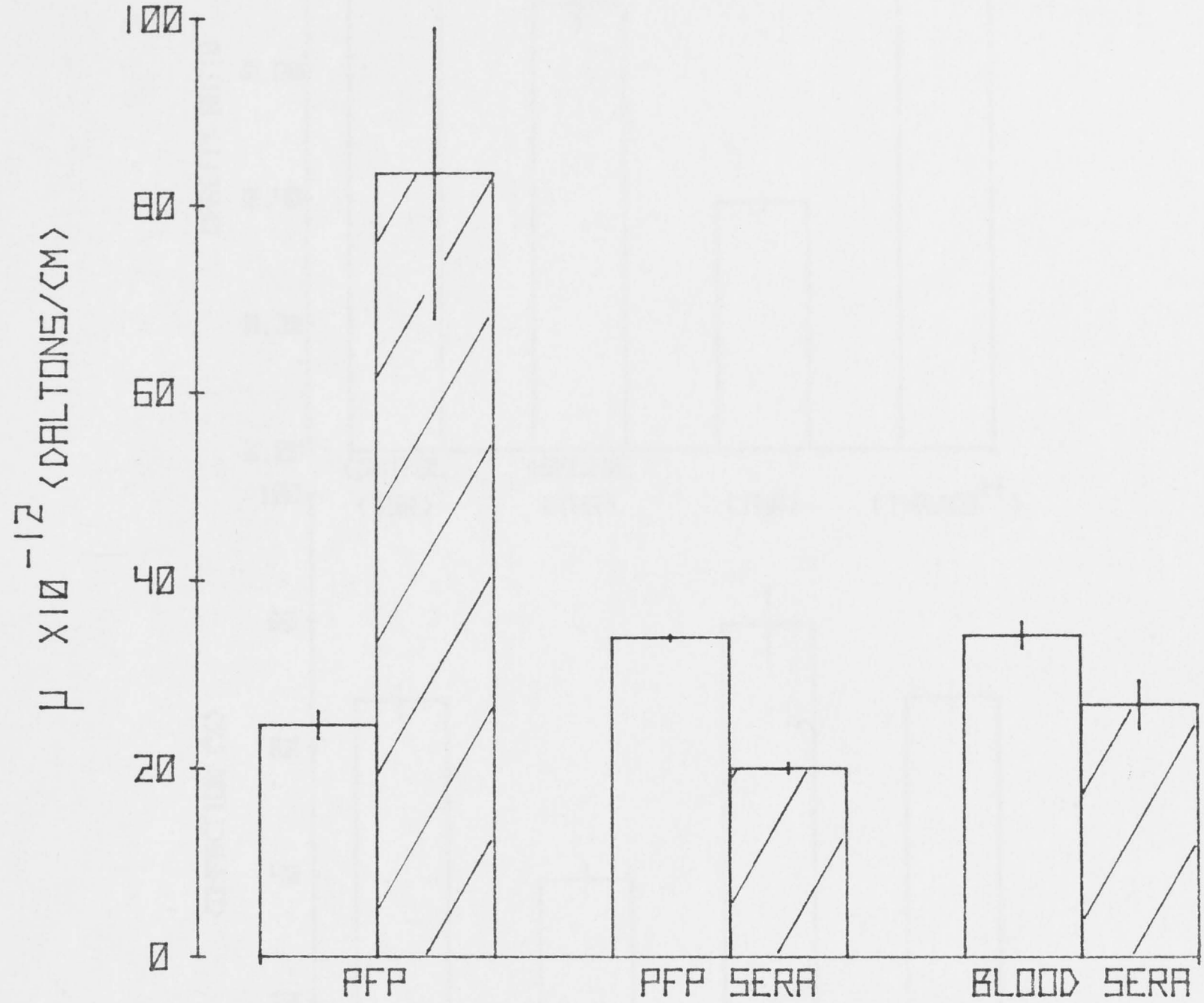


Figure 8.3 The μ_T and μ_p of networks formed in PFP and reconstituted sera

The μ_T (open bars) and μ_p (cross-hatched bars) was determined in networks made in PFP or in the sera from PFP (PFP Sera) and blood (Blood Sera) which was reconstituted with fibrinogen. Results are the mean of data from 5 observations $\pm$ SEM.

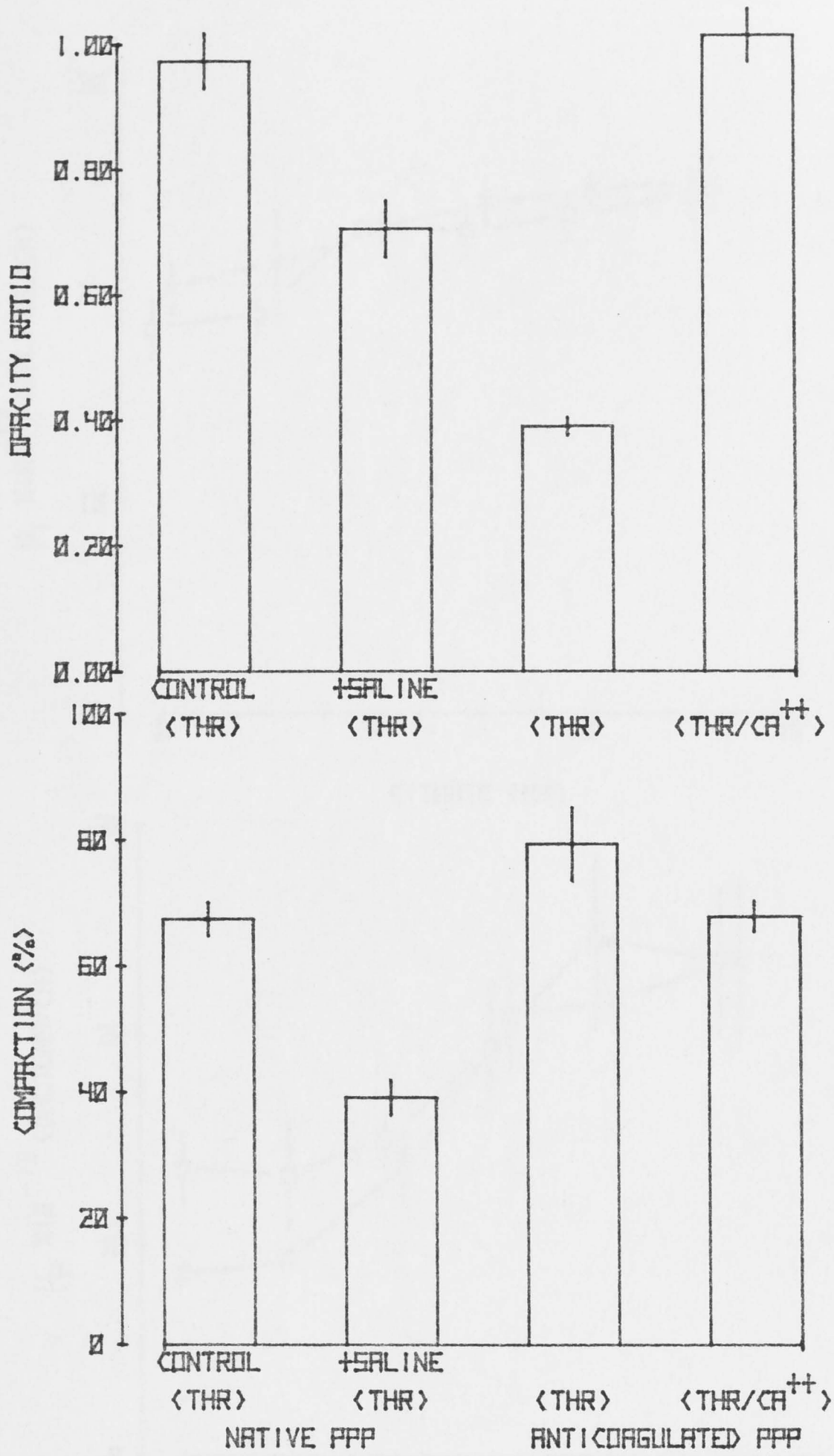
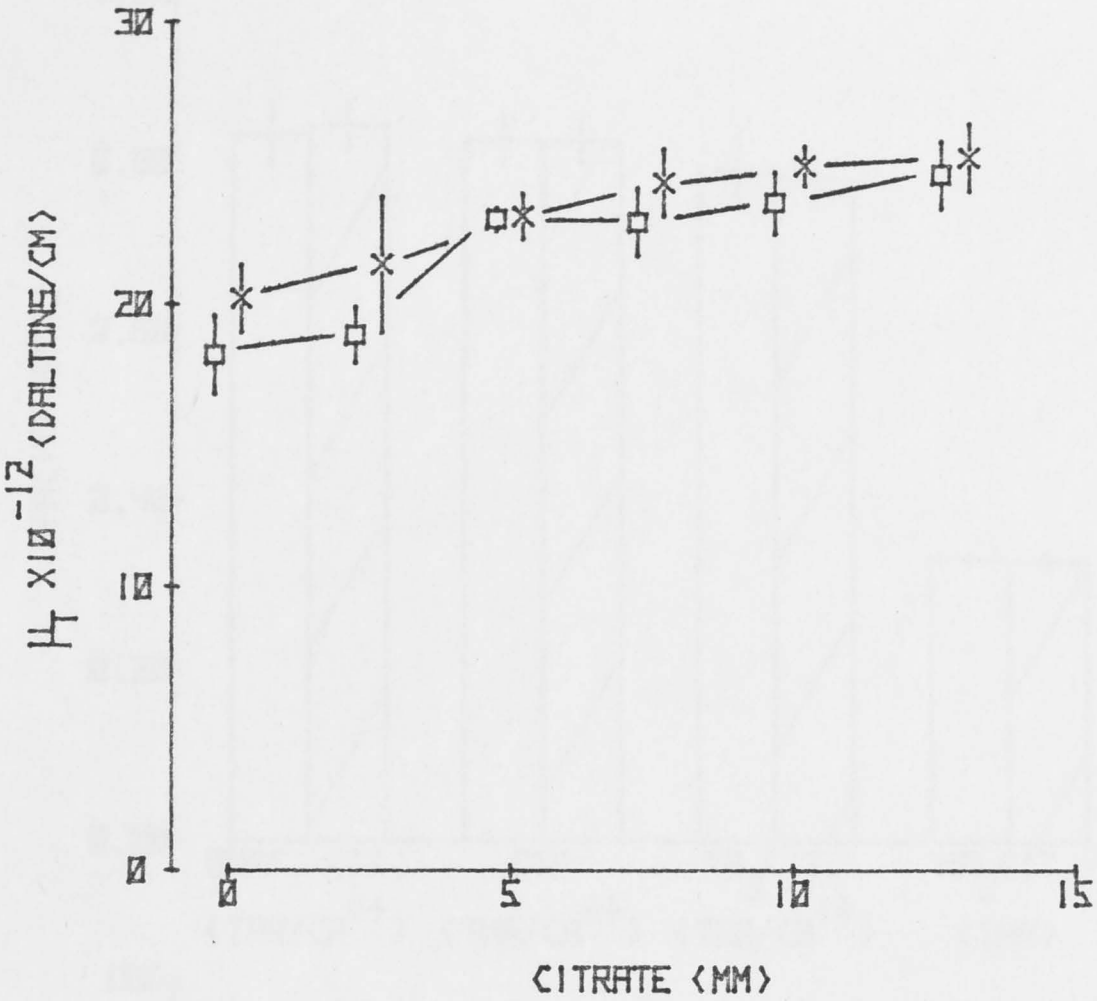


Figure 8.4 The effect of anticoagulant and recalcification on opacity ratio and compaction

Networks were formed in PPP prepared with and without anticoagulant and clotted with thrombin/$CaCl_2$ (THR/CA^{++}) or thrombin alone. The native PPP either did not contain any additives (CONTROL) or it contained NaCl of the same ionic strength as the anticoagulant (+ SALINE). Results are the mean of 10 observations $\pm$ SEM.



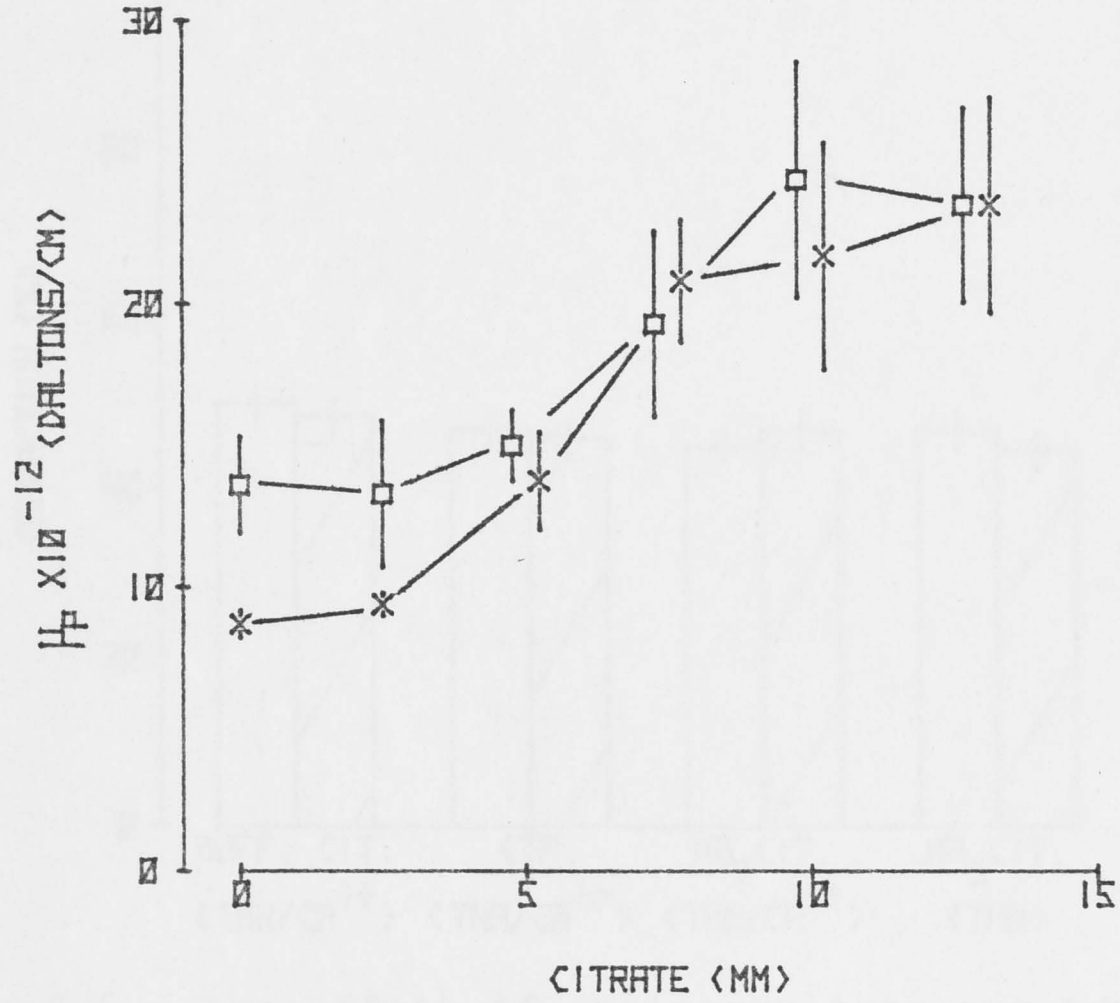


Figure 8.5 The effect of citrate on μ_T and μ_p

Networks were formed in fibrinogen solutions (3.3 mg/ml) which either did not contain divalent cations (□) or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (X). Results are the mean of 3 observations ± SD.

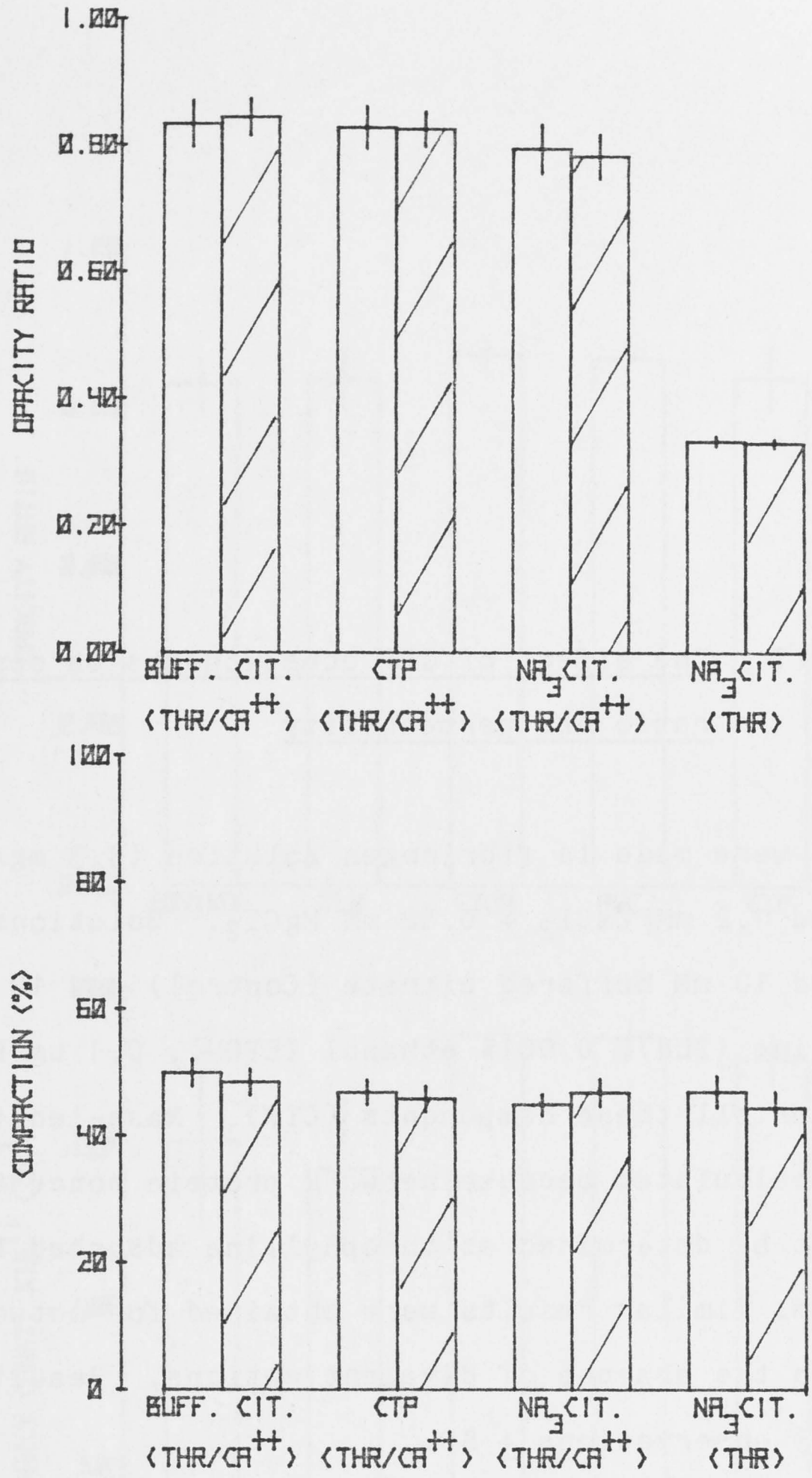
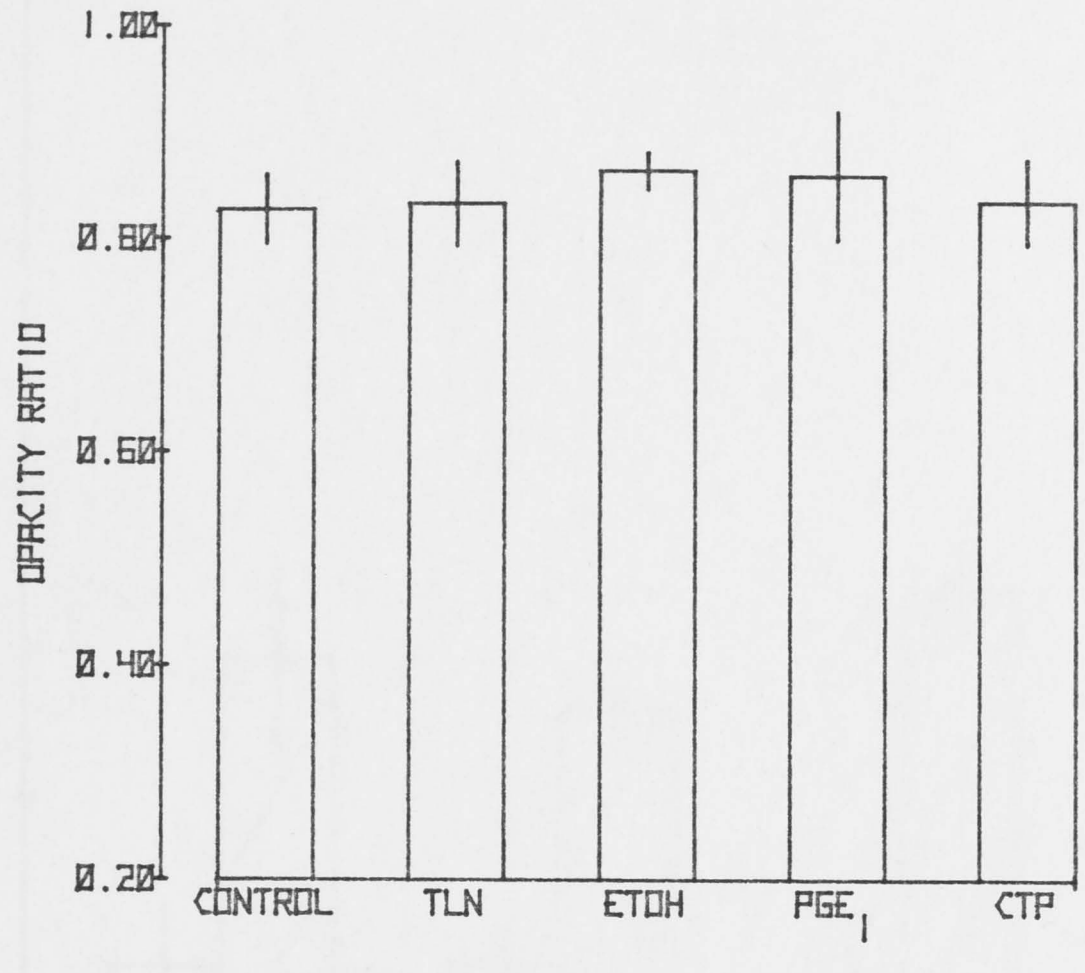


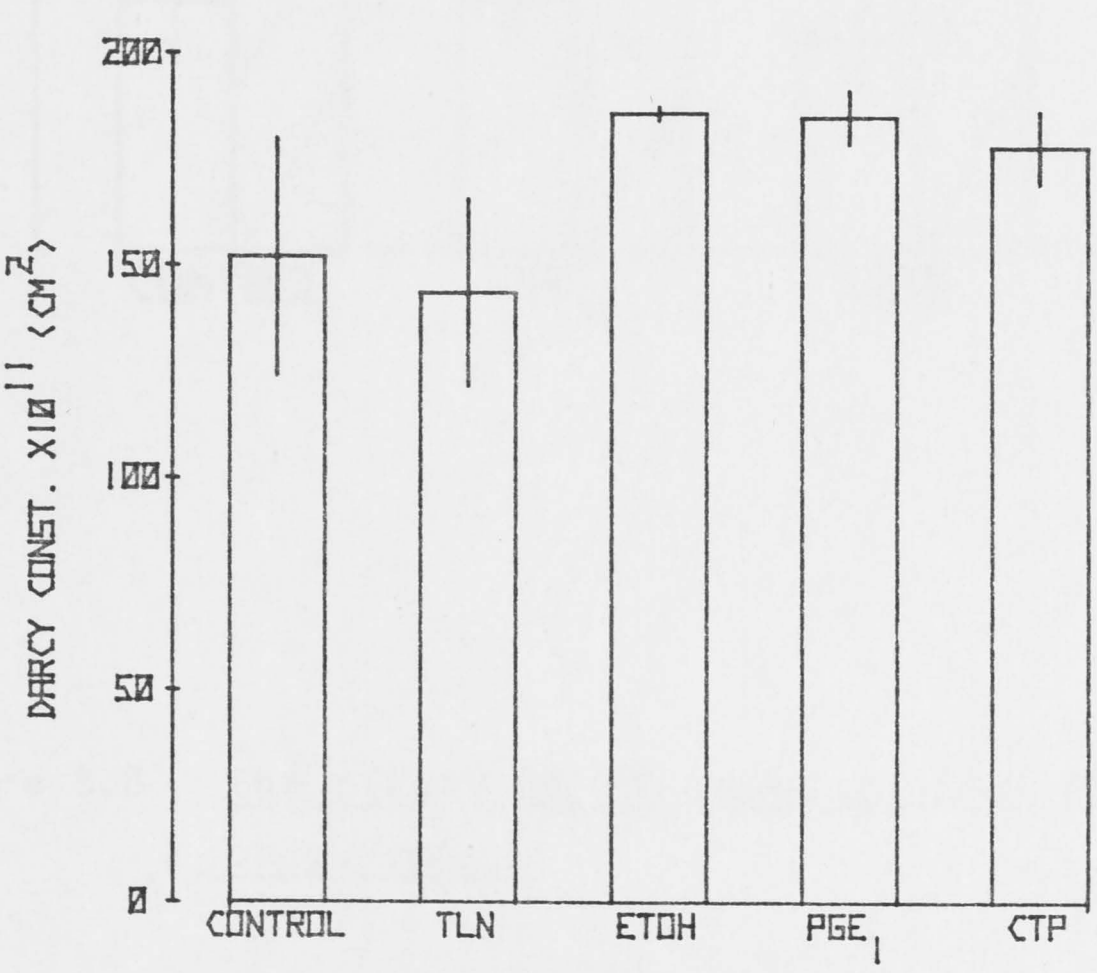
Figure 8.6 The effect of anticoagulants and temperature on opacity ratio and compaction

Networks were formed in PFP prepared with buffered citrate (BUFF. CIT.), CTP or 3.8% trisodium citrate (NA_3 CIT) at room temperature (crosshatched bars) or on ice (open bars) and clotted with thrombin/$CaCl_2$ (THR/CA^{++}) or thrombin alone (THR) at room temperature. Results are the mean of 10 observations $\pm$ SEM.

Figure 8.7 The effect of CTP constituents on opacity ratio and permeability

Networks were made in fibrinogen solution (3.3 mg/ml) which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. Solutions also contained 10 mM buffered citrate (Control) and 10 mM theophyline (TLN), 0.001% ethanol (ETOH), 0.1 ug PGE_1 (PGE_1), or all these components (CTP). Mass-length ratio was not calculated because network protein concentration could not be determined as theophylline adsorbed strongly at 282 nm. Similar results were obtained for networks formed in the absence of divalent cations. Results are the mean of 3 observations $\pm$ SD.


1.00
0.80
0.60
0.40
0.20
OPACITY RATIO
CONTROL
TLN
ETOH
PGE1
CTP


200
150
100
50
0
DARCY CONST. X10^11 (CM^2)
CONTROL
TLN
ETOH
PGE1
CTP

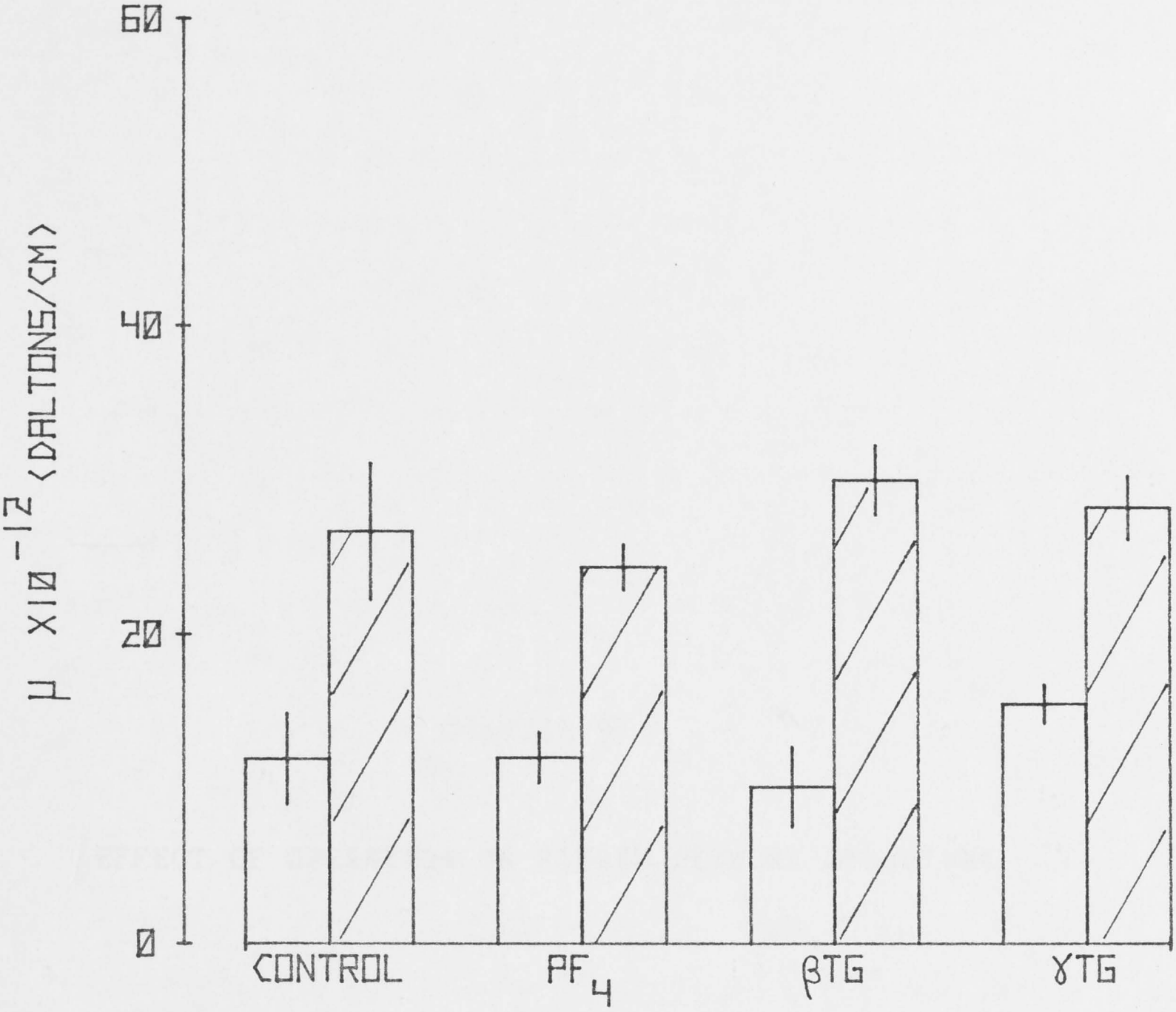


Figure 8.8 The effect of 100 μg/ml of PF_4, βTG and γTG on μ_T and μ_p

Networks were formed in fibrinogen solution (1.5 mg/ml) which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. Similar results were obtained in the absence of divalent cations. The open bars represent u_T; the cross-hatched bars represent u_p. Results are the mean of 3 observations ± SD.

CHAPTER 9

EFFECT OF OPERATION ON FIBRIN NETWORK STRUCTURE

9.1 INTRODUCTION

The postoperative period is characterized by a number of changes in the haemostatic mechanism: the platelet count decreases immediately after surgery (Innes and Sevitt, 1964), platelet adhesiveness increases (Ardlie et al., 1967), whole blood clotting time and recalcified plasma clotting time shorten (Blomback et al., 1964), fibrinogen concentration increases (Egeberg, 1962) and fibrinolytic activity decreases (Innes and Sevitt, 1964). It seemed possible that this period is also associated with changes in fibrin network structure and this has been examined.

9.2 MATERIALS AND METHODS

Blood was obtained from 26 patients admitted to the Woden Valley Hospital for elective abdominal surgery preoperatively, and then on the first, third and fifth days following surgery. Informed consent was obtained from all patients. Those who had taken aspirin or other drugs known to affect platelet reactivity or blood coagulation were excluded from the study.

Blood obtained with minimal stasis with a 19G needle was collected in the ratio of 9 : 1 in 3.8% trisodium citrate. It was centrifuged at room temperature for 15 minutes at 1,300g to obtain platelet-poor plasma (PPP). Plastic or siliconized glassware was used for handling blood and plasma.

900 µl of PPP was clotted with 100 µl of 2.5 U/ml thrombin and 30 minutes later, the turbidity at 350 and 608 nm was recorded using plasma as the blank. Turbidities were used to calculate opacity ratio and μ_T (Chapter 2). PPP was also diluted 1 in 3 volumes with 0.895% NaCl. 900 µl of this diluted PPP was clotted with 100 µl of 2.5 U/ml thrombin and the compaction, opacity ratio and μ_T were determined as fully described in Chapter 2. The fibrinogen concentration of plasma was determined gravimetrically (Hickman, 1971). The refractive index of plasma in healthy subjects (Appendix 1) was used to calculate μ_T from turbidity and fibrin concentration.

Fibrinogen to fibrin conversion data in healthy subjects was used to calculate μ_T. The amount of fibrinogen converted into fibrin in diluted and undiluted PPP samples from 10 donors was determined by adding ^{125}I fibrinogen to the plasma before clotting. Plasma was clotted as described above and centrifuged at 30 minutes to obtain serum. Radioactivity of the serum was measured and the percentage conversion calculated.

9.3 RESULTS

Fibrinogen concentration, compaction (Figure 9.1), opacity ratio and μ_T (Figure 9.2) in fibrin network increase significantly after operation. There is a further increase on the third postoperative day in all values with the exception of μ_T. This is followed by a return

towards preoperative values on the fifth postoperative day. The μ_T returned towards preoperative value after the first postoperative day and remained at preoperative levels on the third and fifth postoperative days. In other words, fibrinogen concentration, compaction and opacity ratio in fibrin network increase substantially following major surgery, but over the same period the mass-length ratio changes relatively little.

Dilution of plasma always resulted in an increase in μ_T, which in diluted PPP was linearly related to that in undiluted PPP (Table 9.1). Whereas less than half of the fibrinogen in undiluted PPP (42.4 $\pm$ 4.2%) was converted to fibrin, nearly all the fibrinogen in diluted PPP (95.3 $\pm$0.6%) was converted to network. The final fibrin concentration in both diluted and undiluted plasma clots did not interfere with the calculation of μ_T.

As shown in Table 9.1 the μ_T and the opacity ratio were linearly correlated with the fibrinogen concentration. Mass-length ratio was not found to be directly related to opacity ratio but when opacity ratio was divided by fibrinogen concentration to allow for changes in the latter, then that value correlated linearly with the mass-length ratio. Neither the fibrinogen concentration nor the mass-length ratio correlated with network compaction.

9.4 DISCUSSION

In this Chapter it has been shown that surgery is followed by consistent changes in fibrin network characteristics. On the first three days after surgery there is a substantial increase in the fibrin mass of the network, that is, in the density of fibres in the network. This is associated with an increase in the ease with which the network may be compacted (Figure 9.1). These parameters begin to return to preoperative values after the third postoperative day. Immediately following surgery μ_T increases and then rapidly returns to preoperative value by the third postoperative day. These findings show that the number as well as thickness of individual fibrin fibres increase following surgery.

The consistency of the postoperative response of fibrin network suggests that it is subject to a regulatory control. But the precise mechanism which underlies the changes described in this Chapter remains unknown. From a comparison of findings in plasma and *in vitro* experiments in purified fibrinogen solutions described earlier in this thesis a tentative understanding of some determinants of fibrin network structure in the postoperative period may be reached.

Firstly it seems likely that compaction in fibrin networks in plasma is not influenced by the postoperative changes in fibrinogen concentration since the two do not

correlate closely (Table 9.1). Furthermore, while compaction increases in parallel with fibrinogen concentration in post-operative plasma (Figure 9.1), in a purified fibrin network compaction decreases when fibrinogen concentration increases (Chapter 6).

Secondly, compaction in networks in plasma does not seem to be influenced by the changes in mass-length ratio because network compaction in plasma did not correlate linearly with μ_T (Table 9.1). This is contrary to findings in purified fibrinogen (Chapter 5).

It seems likely that changes in the tensile properties of fibrin and to a lesser extent, changes in the distribution of fibres in the major and minor networks underlies the observations made on compaction of fibrin networks in plasma in the postoperative period.

Thirdly, while the mechanism underlying the initial increase in μ_T on the first postoperative day is not clear the decrease towards preoperative values on the third and fifth postoperative days may be related to the increase in the fibrinogen concentration. It is known that an increase in fibrinogen concentration above physiological levels decreases μ_T (Chapter 6). Indeed, μ_T was inversely related to fibrinogen concentration in the post-operative period (Table 9.1).

Fourthly, it would seem that the changes in opacity

ratio result from an increase in the plasma fibrinogen after operation. The statistical correlation between opacity ratio and fibrinogen concentration is highly significant (Table 9.1). Furthermore, a strong influence of fibrinogen concentration on opacity ratio is revealed in the linear correlation between μ_T and opacity ratio divided by fibrin concentration (Table 9.1). This supports earlier findings (Chapter 5) which show that opacity ratio is influenced by both μ_T and the density of network fibres.

9.5 CONCLUSIONS

(1) The structure and properties of fibrin networks in plasma changes consistently after an operation. Following surgery, network compaction and fibrinogen content increase substantially whereas μ_T increases only transiently.

(2) Unlike compaction in networks made in purified fibrinogen solutions those in plasma are not directly influenced by changes in fibrinogen concentration or by fibre thickness.

(3) Results suggest that fibrinogen concentration is a major factor affecting post-operative changes in μ_T in plasma clots.

Table 9.1 The linear correlations between sets of data expressed as correlation coefficient and level of significance.

PLOT OF:	CORRELATION COEFFICIENT (r)
μ_T^1 vs u_T^2	0.737***
μ_T^1 vs opacity ratio	-0.063 NS
μ_T^1 vs fibrinogen (mg/ml)	-0.480***
opacity ratio vs fibrinogen (mg/ml)	0.801***
μ_T^1 vs opacity ratio/(mg/ml)	0.649***
μ_T^2 vs compaction	0.187 NS
compaction vs fibrinogen (mg/ml)	0.134 NS

Data are from all 26 patients on 4 days (n = 104). *** indicates significance at greater than $p(F) < 0.0001$ while NS indicate $p(F) > 0.05$. Superscript 1 and 2 refer to data from undiluted and diluted plasma, respectively.

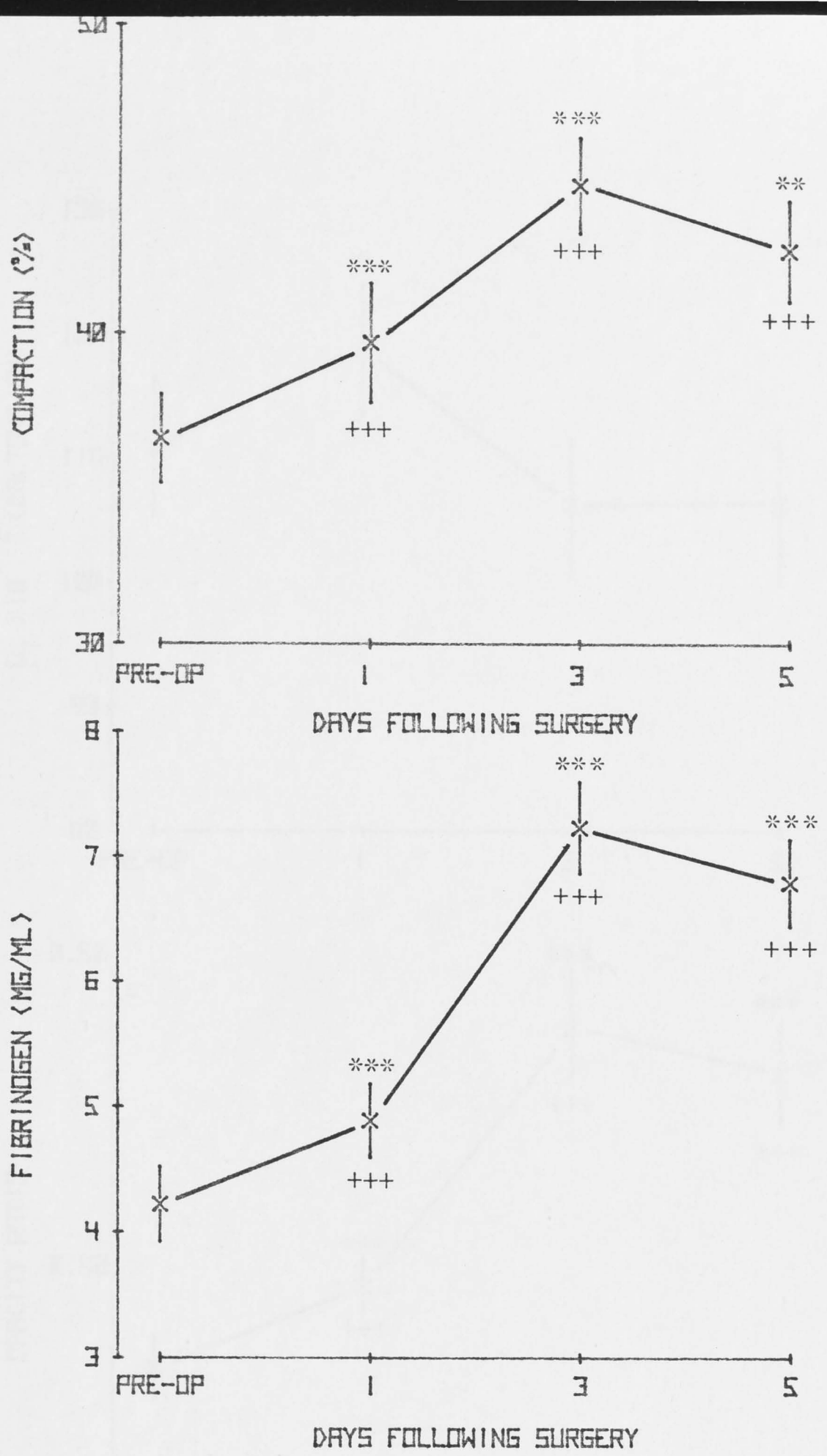
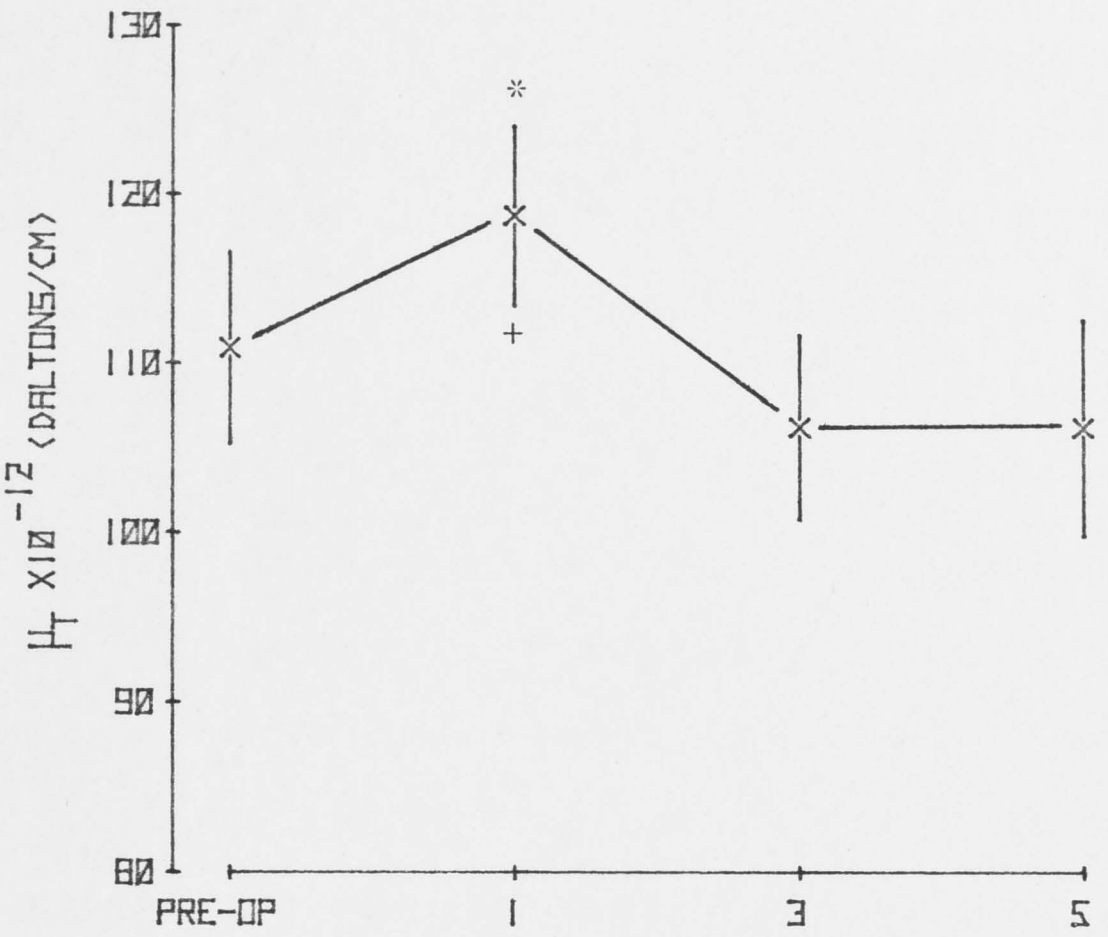


Figure 9.1 The changes in compaction and fibrinogen concentration following surgery.

Compaction data are from networks formed in diluted plasma. Results are the means ± SEM of 26 patients. *,** and *** represents results significantly different from data from the preceding study day at the 0.05, 0.005 and 0.001 level (paired t test). +, ++ and +++ represents results significantly different from the preoperative values at 0.05, 0.005 and 0.001 level (paired t test).



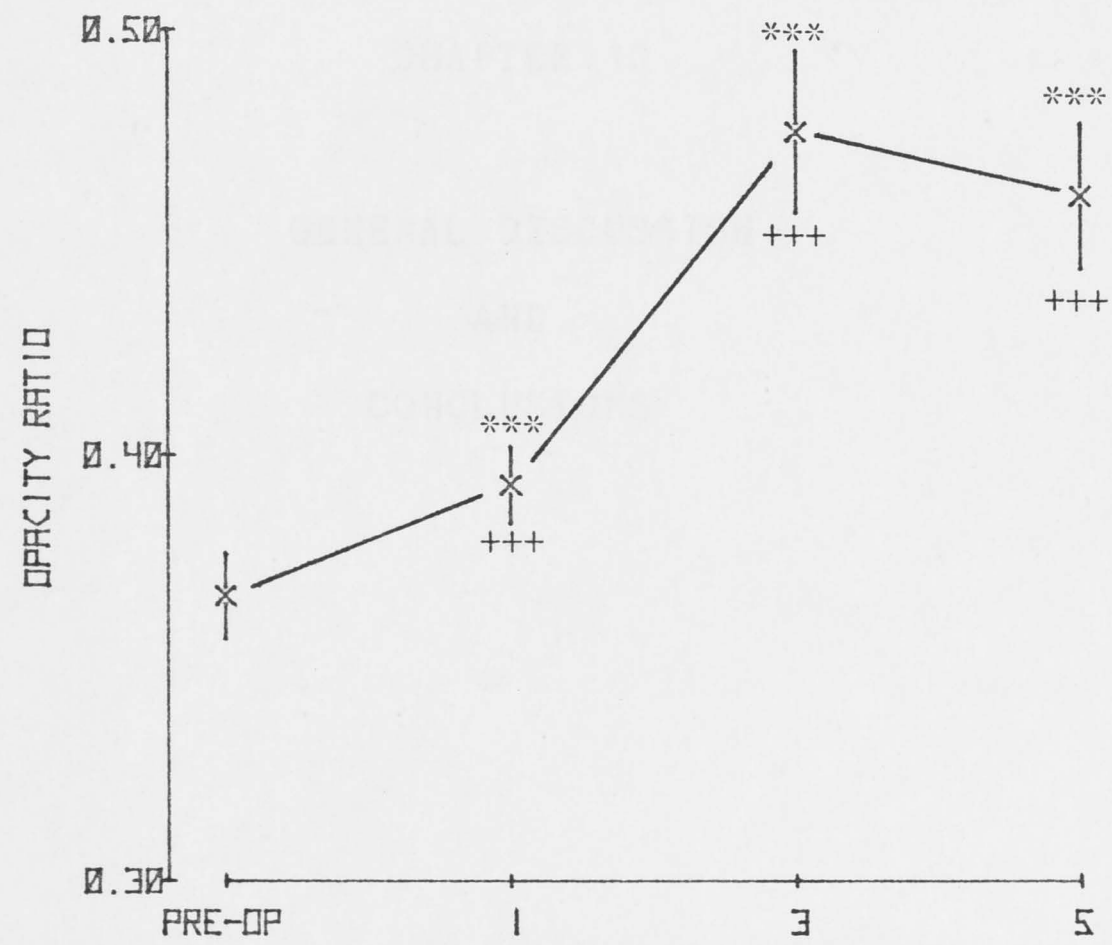


Figure 9.2 The changes in μ_T and opacity ratio following surgery.

Data are from networks formed in undiluted plasma. Other caption details as in Figure 9.1.

CHAPTER 10

GENERAL DISCUSSION

AND

CONCLUSIONS

10.1 GENERAL DISCUSSION

It seems surprising that although a great deal of attention has been focused on the dynamics of the coagulation of blood in particular on the clotting time, the end product of the coagulation cascade, the fibrin network, has not been as critically examined. Recent technological advances have allowed development of automated turbidimetric techniques for determining the clotting end point. Routine clinical use of such techniques has demonstrated that in plasma clots turbidity can vary considerably. Turbidity reflects network structure. The general assumption that in any clot formed under physiological conditions the network of fibrin fibres is much the same is not true. The general aim of the investigation described in this thesis was to examine the variation in fibrin network characteristics in clots made under physiological conditions.

As a background to the studies described in this thesis it will be recalled that more than three decades ago it was shown that fibrin clots of radically different turbidity and mechanical properties may be formed by altering the composition of the clotting mixture (Ferry and Morrison, 1947). Furthermore, through *in vitro* studies it was established that the fibrin networks vary continuously between two extreme types - fine and coarse and that variation in the thickness and number of fibres in a network are responsible for the variation in clot

characteristics. However, much of the early work of fibrin networks as well as a good deal of the more recent studies on the mechanism of fibrin polymerization (Carr et al., 1977; Rosser et al., 1977; Carr and Hermans, 1978; Hantgan and Hermans, 1979) concentrated on networks formed under non-physiological conditions. The relevance of previous observations to the clinical situation has remained unclear, a major obstacle being the lack of suitable methods applicable to sequential studies in clinical settings.

The several methods available for studying fibrin network structure are not only based on independent network characteristics but have been developed more or less independently of each other for the study of networks formed under non-physiological conditions. It is important at first to examine the applicability of these methods to networks formed under physiological conditions (Chapter 3). A methodological study was conducted to determine whether changes in network characteristics recorded with one methods may be transcribed in terms of another method, that is, whether a single method is capable of providing all the relevant information about fibrin network structure. However, this was found not to be the case. Thus, for example, although a mathematical relationship between lag time and network permeability had previously been postulated (Blomback and Okado, 1982a) it was shown in Chapter 5 that this relationship holds only under certain conditions. It was concluded that the various methods

cannot replace each other. Since the characteristic upon which the different methods are based overlap to some extent, the various methods complement each other, each method providing information unique to itself and extending that obtained using the other techniques. Thus, for instance, information regarding the three dimensional arrangement of fibres in the network is provided by scanning electron microscopy; that regarding the distribution of fibre sizes by transmission electron microscopy. Measurement of pore size and average mass-length ratio of thinner network fibres may be made with permeability technique while the average mass-length ratio of thicker fibres may be derived from turbidity methods. The opacity ratio technique makes a measurement of fibre thickness and fibre density while the compaction technique provides information on fibre thickness, density and tensile characteristics. Kinetics of network development may be analysed from the lag time and ΔOD/sec using the turbidity technique. Thus, maximal information is obtained by using concommittantly a variety of methods so that a composite picture of fibrin network may be built up and this approach has been adopted in the studies described in this thesis.

It was found that turbidity measurements are sensitive predominantly to the thicker fibres in a network whereas permeability and compaction are influenced by the size of spaces between network fibres, that is, by the more numerous thinner network fibres. Since measurement of

network permeability and compaction involve physical manupulation of the network, they are sensitive to factor $XIII_a$ mediated crosslinkages. Not so the turbidity method. It should be noted that measurements of permeability, compaction and turbidity are all influenced by the fibrin content of the network and an allowance should be made for fibrin content in opacity ratio and compaction. Thus, these methods are sensitive to fibrin fibre density in addition to other characteristics of the network. These observations described in the earlier part of the thesis not only underlie the limitations of the various methods but also indicate that network characteristics are modified by factor $XIII_a$ mediated crosslinking, the fibrin content, the range of fibre sizes in the network as well as by the average thickness of the network fibres.

It has previously been generally assumed that fibrin networks are composed of fibres of uniform size. This assumption has been shown to be untrue. It was shown in Chapter 4 that fibrin fibres are, in fact, organized into two intimately associated networks, a major and a minor network. The major network, which is formed from the bulk of the fibrinogen, is composed of the thicker fibres. The fibre thickness in the major network, however, is not uniform. The minor network is located within the spaces between the major network fibres. It is a mesh of very thin and numerous fibres which arise from a relatively small mass of the total fibrin within the network. When

fibrin is stabilized by factor $XIII_a$ mediated crosslinkages the minor network is anchored within the major and this alters the properties of the network as a whole.

The bimodality of fibre distribution accounts, in part, for the differences in network structure recorded with different methods. As has been shown theoretically (Appendix 5) and experimentally (Blomback and Okado, 1982b) that minimum pore size in fibrin networks is much smaller than the average pore size calculated using permeability. The average pore size and hence μ_p, is reduced in crosslinked fibrin networks as the minor network offers a greater resistance to perfusion. In non-crosslinked networks, however, the minor network does not offer similar resistance to flow and therefore the average pore size and μ_p represents that of the major network. Turbidity measurements and μ_T do not depend upon the minor network nor on whether the fibres of this network are crosslinked or not. Because turbidity and permeability measuremments are sensitive to opposite ends of the fibre size spectrum the μ_T provides a measure of the average mass-length ratio of the thicker network fibres of the major network whereas μ_p provides the average mass-length ratio of the thinner network fibres.

Crosslinking by factor $XIII_a$ stabilizes the minor network. This results in a reduction in network permeability and compaction but not at the expense of the

elastic modulus of the network which in fact increases (Kamykowski et al., 1981). In contrast, the elastic modulus decreases with permeability and compaction if these are lowered by reducing the average mass-length ratio of the network (Kamykowski et al., 1981). Since the mechanical characteristics of fibrin networks are undoubtedly important in haemostasis it seems likely that factor $XIII_a$ plays a vital role in stabilizing the minor network and hence in modifying the mechanical characteristics of the fibrin network.

The studies in Section I of this thesis make it clear that the proportion of fibrin in the major and minor networks and the distribution of fibre sizes in these networks is as important to the complete description of fibrin network structure as is the average fibre thickness and fibre density (and perhaps the density of network fibre branch points - Mueller and Birchard, 1978). At present little is known about the regulation of fibrin distribution in the major and minor networks and this is clearly an area for further research.

Although the distribution of fibre sizes and the proportion of fibrin in the major and minor networks is important to a complete description of fibrin network structure, some evidence obtained in Section II of this thesis supports the use of a less detailed description. The shape of the distribution of fibre sizes in the network might not alter greatly as μ_p and μ_T, which are

sensitive to opposite ends of the fibre size distribution, generally changed in parallel. Furthermore, the proportion of fibrin in the major and minor networks also might not vary greatly as the μ_p of crosslinked networks (which is sensitive to the minor network) generally changed in parallel with the μ_p of non-crosslinked networks (which is apparently insensitive to the minor network). For these reasons, a generalized model of fibrin network structure varying in fibrin content and average mass-length ratio may be used to examine the mechanism underlying changes in physiological fibrin network structure.

It is clear that fibrinogen concentration is an important determinant of the density of fibres in the network and hence network properties and structure. In Section II it is shown that an increase or decrease in fibrin concentration causes a parallel change in fibre density. However, these two are only directly related when fibre thickness does not change. When fibre thickness changes even at a fixed fibrinogen concentration, the fibre density must change proportionally. It is, thus, clear that an understanding of how fibre thickness is altered is of paramount importance in understanding the regulation of network structure and properties.

Studies in Section II of this thesis allow some understanding of the mechanism underlying changes in average fibre density and fibre thickness. It should be noted that this area of investigation has been relatively

neglected since Ferry and Morrison (1947) postulated that differences in the activity coefficient of fibrinogen cause differences in fibrin network structure. More recent work has suggested that fibrin networks are metastable structures. Fibrils are inhibited from associating laterally with each other when the density and interpenetrating nature of the fibres in the network is such that their rotational diffusion is inhibited (Nelb et al., 1976). Until this stage of network development is reached the diffusion of fibrils enhances the extent of lateral aggregation and hence increases the mean network fibre thickness. Hantgan and Hermans (1979) used this concept to explain the relationship between changes in the kinetics of fibrin generation and changes in network structure.

These concepts on the mechanism underlying changes in network structure may be unified and extended on the basis of observations on physiological fibrin networks. It was noted that the kinetics of fibrin generation changes only slightly when the mass-length ratio was altered substantially by adding substances which strongly affect fibrin(ogen) solubility (e.g., heparin, protamine sulphate). On the other hand, a substantial change in the kinetics of fibrin generation (in terms of lag time) affected by slightly modifying thrombin or fibrinogen concentration causes minor changes only in the mass-length ratio. Moreover, although divalent cations increase mass-length ratio they do not significantly influence changes in

network structure caused by other factors. It, therefore, appears likely that the solubility of fibrin(ogen) (which is related to the activity coefficient) primarily determines the rate of lateral association of fibrin fibrils while the kinetics of fibrin generation primarily influences the extent of lateral association by controlling the time available for fibril rearrangement.

The classification of factors which modify network structure in terms of two characteristics (*viz* factors which either predominantly affects fibrin(ogen) solubility or factors which predominantly modify kinetics of fibrin generation) makes it relatively easy to identify potential influences on fibrin network structure. Using these criteria we can predict that reduced fibrin generation (as in haemophiliac plasma) should result in fibrin networks of abnormally high mean fibre thickness and this has been found to be the case (Bettingle *et al*., 1964). Likewise, substances which reduce the solubility of fibrin(ogen) through steric exclusion effects, such as dextran (Rampling *et al*., 1976) should increase mean fibre thickness. This has also been shown (Dhall and Bryce, 1970). Furthermore, as fibrin network structure and properties in both purified fibrinogen solution (Chapters 6 and 7) and in plasma (Chapter 8) are sensitive to non-specific changes in clotting conditions it would seem that networks formed *in vivo* may also be sensitive to therapeutically induced or spontaneous changes in clotting conditions.

The importance of sensitivity of network structure *in vivo* would be a moot point if fibrin formed only where homeostasis ensured minimum fluctuation in plasma composition. But since fibrin serves a reparative function it is formed where haemostatic mechanisms may not adequately control factors such as ionic strength, pH and temperature. In addition, the local variation in concentrations of glycosaminoglycans and certain cellular components (e.g., histones - Kopec *et al*., 1974) as well as thrombin and fibrinogen may significantly affect final network structure. Since certain bleeding disorders are associated with the development of fibrin networks of abnormal structure and properties (Shaw, 1980) it seems likely that variation in network characteristics may be important physiologically.

Although abnormal fibrin network structure and properties have been identified clinically through comparison of network formed in plasma little was known about the factors which influence network structure. Indeed, it was not even known how networks in purified fibrinogen solution differ from those in plasma. This was examined in Section III of this thesis.

The choice of anticoagulant and the handling procedures used in preparing and clotting the plasma were shown to affect network structure (Chapter 8). The anticoagulant was found to be a major determinant of ionic strength and pH of the plasma and hence it was capable of modifying

network structure. Anticoagulants also inhibited fibrin formation in plasma but this could be nullified by recalcification of the plasma. If the plasma was recalcified care had to be taken to remove all platelets to prevent significant network retraction. These studies indicated that strict standardization of methodology is essential for routine clinical studies on fibrin networks in plasma.

Apart from the influences of anticoagulants, networks formed in plasma differ from those in purified fibrinogen solutions because they are formed in the presence of significant amounts of fibronectin and α_2-antiplasmin which are incorporated into the network (Mosher, 1980). It was shown that networks formed in plasma are more permeable and deform more readily than those in purified fibrinogen solutions. The reasons for these differences remain unclear. However, since networks in reconstituted sera did not exhibit the enhanced permeability of networks in plasma it seems possible that some plasma component is responsible and that it is in some way consumed during network development.

Although platelets are known to contain substances which reduce mean network fibre mass-length ratio (Dhall et al., 1982), the cellular elements in blood were shown not to release stable serum soluble factors which increase the mass-length ratio. It thus seems likely that the very thick fibrin fibres found associated with platelets in

thrombi (Gottlub, 1975; Hattori et al., 1978; Hisano, 1978) are not caused by enhanced lateral polymerization due to platelet release products, but rather they may represent thinner fibres drawn together during platelet mediated clot retraction (James et al., 1960; 1962; Szalontai, 1968).

The final Chapter in this thesis examined fibrin networks in a clinical setting. The effect of surgical trauma on fibrin network structure was examined using sequential measurements (Chapter 9). It was found that network structure changes characteristically following an operation. The fibre density and the fibre mass-length ratio increase immediately after surgery. But while fibre density continued to increase until the third postoperative day becuase of the increase in plasma fibrinogen levels, the mass-length ratio quickly returned to preoperative levels. This decrease in mass-length ratio correlated with the increase in fibrinogen concentration but the cause of the initial increase in mass-length ratio is unknown. It was noted that networks compacted more easily following surgery. This increase in compaction paralleled the increase in fibrinogen level but was not related to either changes in mass-length ratio or fibrinogen concentration. It, therefore, seems likely that this behaviour, which is opposite to what might be expected from studies using purified fibrinogen (Chapter 6), may be the result of changes in the tensile characteristics of the network.

From this clinical study it is clear that network

structure and properties are subject to some form of physiological control or regulation. Changes in fibrinogen level (that is, in the kinetics of fibrin generation) play some part in this control. More detailed studies are needed to identify other influencing factors.

10.2 CONCLUSIONS

Fibrin networks were formed in plasma or in fibrinogen solutions under conditions which simulated those in plasma and these networks were examined with a variety of methods. A critical examination and comparison of the methods was undertaken to establish their suitability to the study of physiological fibrin networks. In addition, influences on fibrin network structure and properties were investigated with the primary aim of deriving information which would support study of fibrin networks in a clinical setting.

It was demonstrated that the measurement of mass-length ratio from permeability but not from turbidity is sensitive to factor $XIII_a$ mediated crosslinking. This finding led to the recognition that crosslinking stabilizes the previously undocumented minor network. This network which is found within the interstitial spaces between the major network fibres reduces network permeability.

The semi-quantitative methods, opacity ratio and compaction were shown to provide a good measure of changes in fibrin thickness but only when the fibrin concentration

was not varied. Compaction was shown to also be sensitive to crosslinking. Measurement of lag time proved to be influenced by changes in network fibre thickness.

The structure and properties of networks formed in fibrinogen solution were shown to be sensitive to small changes in clotting conditions and to the presence of a variety of clinically and physiologically important substances such as heparin, protamine sulphate, glucose and glycosaminoglycans.

Networks formed in plasma were shown to be sensitive to anticoagulants, ionic strength and pH but the effects of anticoagulant could largely be neutralized by recalcification.

Networks formed in plasma differed from those formed in fibrinogen solutions in terms of permeability and ease of deformation. The mass-length ratio of networks formed in plasma was not increased in the presence of platelet release products.

Network structure and properties changed consistently after operation. Fibre density and network compaction increased markedly while mass-length ratio initially increased and then decreased. Postoperative changes in mass-length ratio correlated with changes in fibrinogen concentration.

PERSONAL PUBLICATIONS

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APPENDICIES

APPENDIX I

REFRACTIVE INDEX MEASUREMENTS

Aim

To measure the refractive indicies of fibrinogen solution and plasma so as to enable calculation of μ_T.

Materials and Methods

Measurements of the refractive index of solutions was made at 22°C using a ABBE - refractometer model A calibrated for light of 589.3 nm wavelength. Preliminary studies showed that the refractive indicies of solutions measured at 615 nm was not significantly different from those measured at 589.3 nm and thus, these latter values were assumed to be not significantly different from those at 608 nm.

Results and Discussion

The refractive index of tris-saline was found to be 1.336. This value increased slightly but significantly with fibrinogen concentration. The refractive index of 10 mg/ml fibrinogen in tris-saline was 1.338.

The refractive index of plasma from 10 healthy individuals was 1.347 $\pm$ 0.001 ($\pm$ SD). The refractive index of the serum from these individuals was 1.349 $\pm$ 0.001.

The refractive index of plasma from three individuals did not change significantly following surgery. The refractive index of plasma prepared as in Chapter 8 was not significantly different from plasma prepared as in Chapter 9.

The refractive indices used in calculating μ_T in purified fibrin and in plasma clots were 1.336 and 1.347, respectively. A difference in refractive index of 0.002 affects μ_T by an insignificant 0.2%.

dn/dc is also used in calculating μ_T. A value of 1.001 was used throughout the studies in this thesis. It was calculated from data presented by Carr et al. (1977).

APPENDIX 2

MEASUREMENT OF u_T USING TURBIDITY AT 608 nm AND USING TURBIDITIES AT A RANGE OF WAVELENGTHS

Aim

To establish whether μ_T can be calculated from the turbidity at 608 nm in the presence of physiological level of fibrinogen.

Materials and Methods

Networks were formed using fibrinogen solution (3.3 mg/ml or 1.5 mg/ml) which did not contain divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$. The pH and ionic strength were varied as in Chapter 4. The turbidities of networks were measured at a range of wavelengths from 350 nm to 800 nm and used to determine μ_T (Chapter 2).

Results and Discussion

μ_T can be calculated from the turbidity provided turbidity is directly proportional to λ^{-3} (Carr and Hermans, 1978). This proportionality was found to hold at long wavelengths but to decay substantially at wavelengths shorter than 550 nm particularly at higher and physiological levels of fibrinogen concentration (Figure

A2.1). This finding receives support from Carr and Hermans (1978).

When turbidity is directly related to λ^{-3} μ_T may be calculated from turbidity at a range of wavelengths or at a single wavelength (Carr and Hermans, 1978). Figure A2.2 shows that μ_T calculated from turbidity at 608 nm correlated linearly with that calculated from turbidity at a range of wavelengths. This relationship holds over a wide range of mass-length ratios. These findings indicate the turbidity at 608 nm can be used to calculate μ_T for a variety of network types.

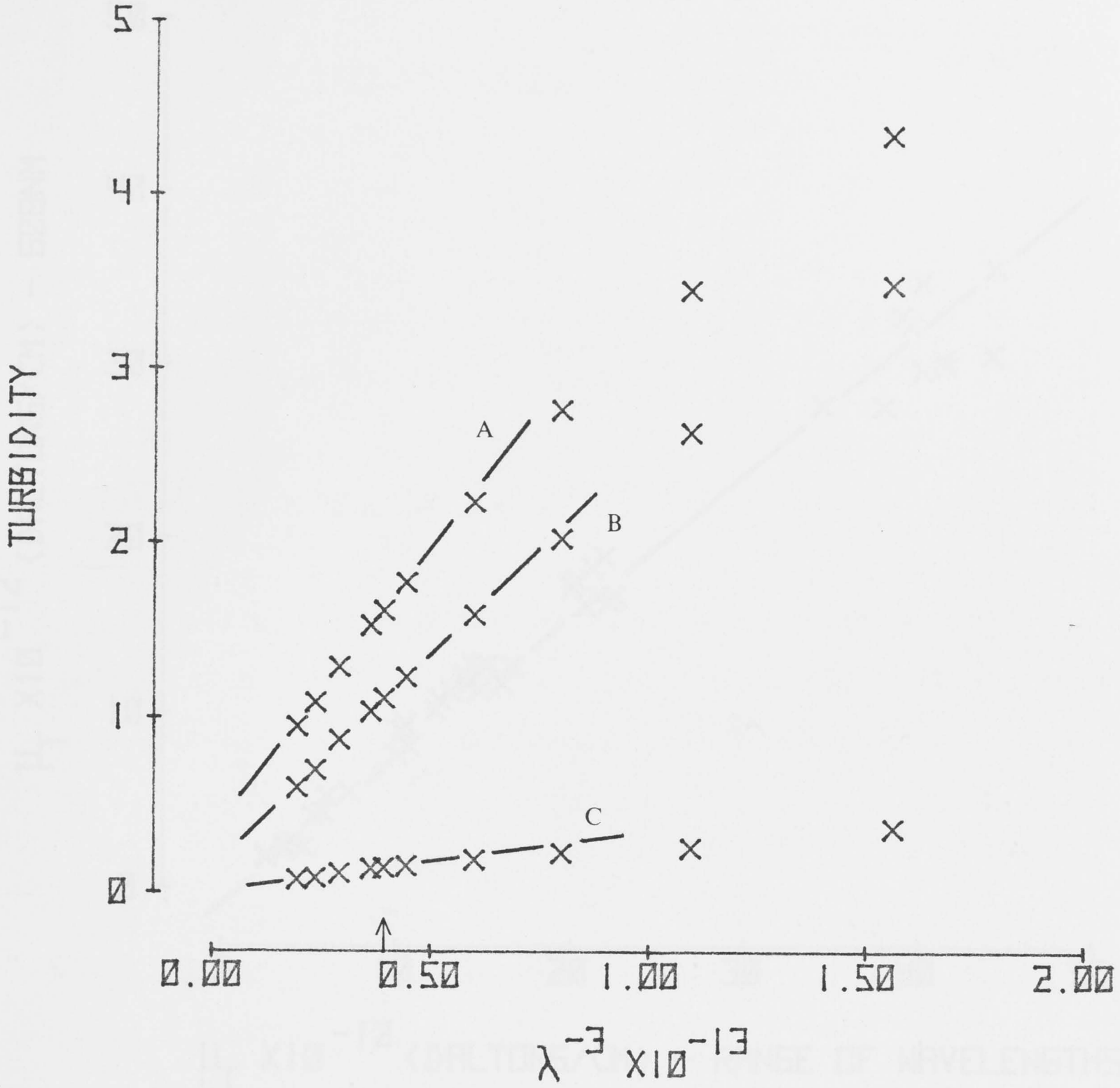


Figure A2.1 The wavelength dependence of the turbidity of different networks

Networks were formed in fibrinogen solutions (3.3. mg/ml) which did not contain divalent cations. Plots A, B, and C were made from fibrinogen solutions of ionic strength 0.103, 0.153 and 0.203, respectively. μ_T equalled 29.7, 12.2 and 1.21 x 10^{12} daltons/cm, respectively. Turbidity data at 608 nm is marked with an arrow.

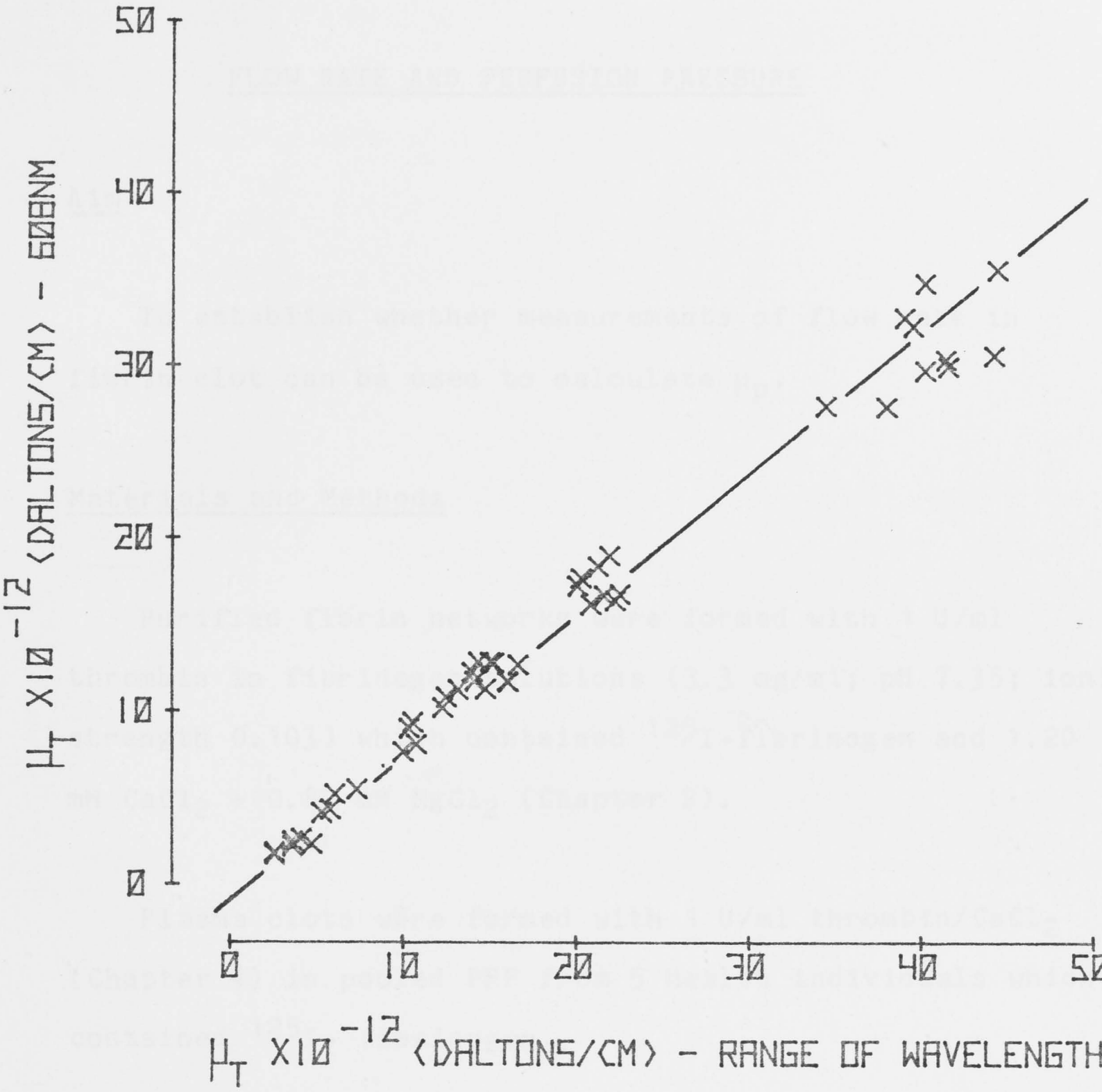


Figure A2.2 Mass-length ratio from turbidity at 608 nm and from turbidities at a range of wavelengths

Networks were formed in fibrinogen solutions (3.3 or 1.5 mg/ml) which did not contain divalent cations or which contained 1.2 mM $CaCl_2$ + 0.82 $MgCl_2$. The pH and ionic strength were varied (Chapter 6). The two mass-length ratio values were significantly linearly correlated ($r = 0.991$; $p(F) < 0.0001$; $n = 44$).

APPENDIX 3

FLOW RATE AND PERFUSION PRESSURE

Aim

To establish whether measurements of flow rate in fibrin clot can be used to calculate μ_p.

Materials and Methods

Purified fibrin networks were formed with 1 U/ml thrombin in fibrinogen solutions (3.3 mg/ml; pH 7.35; ionic strength 0.103) which contained ^{125}I-fibrinogen and 1.20 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (Chapter 2).

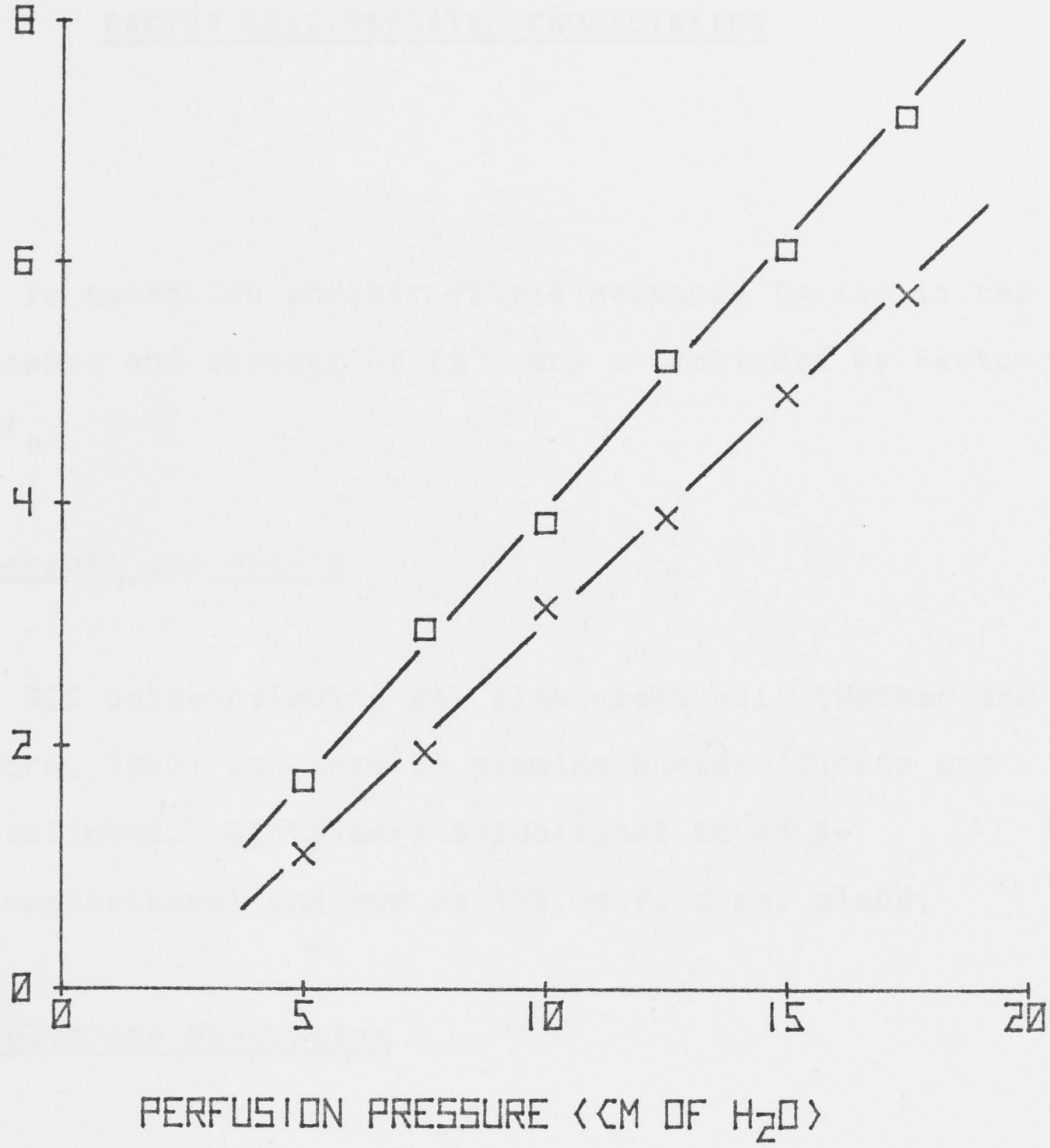
Plasma clots were formed with 1 U/ml thrombin/$CaCl_2$ (Chapter 9) in pooled PFP from 5 health individuals which contained ^{125}I- fibrinogen.

Networks were left to develop fully for 1 hour. The clots were then perfused and the flow rates measured (Chapter 2) at a variety of different pressures.

Results and Discussion

Figure A3.1 shows that flow rate is directly proportional to perfusion pressure in both plasma clots and purified fibrin clots. Thus, flow through fibrin networks

obeys Poiseuille's law. This is supported by previous observations (Blomback and Okado, 1982a). Therefore, the Darcy constant and μ_p may be calculated from flow rate through clots made in fibrinogen solution or in plasma.



Figures A3.1 The relationship between flow rate and perfusion pressure in plasma and purified fibrin clots

Networks were formed in PFP (□) clotted with thrombin/ $CaCl_2$ (Chapter 8) or in purified fibrinogen solution (3.3 mg/ml, pH 7.35, ionic strength 0.103) - (×). Results are the mean of 2 determinations.

APPENDIX 4

FACTOR XIII MEDIATED CROSSLINKING

Aim

To establish whether fibrin networks formed in the presence and absence of Ca^{++} are crosslinked by Factor $XIII_a$.

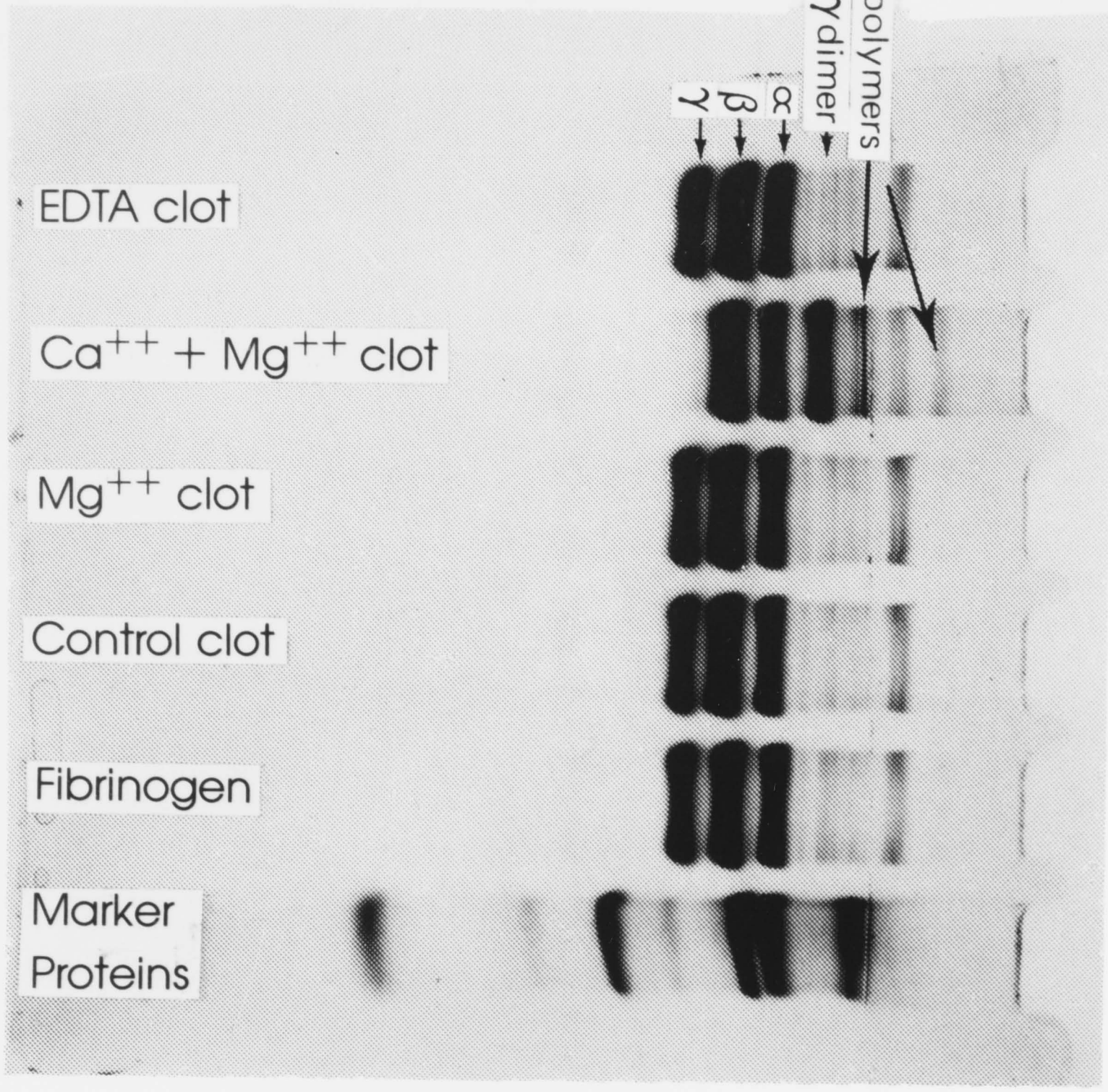
Materials and Method

SDS polyacrylamide gel electrophoresis (Webber and Osborn, 1969) was used to examine whether fibrin was crosslinked. Clots were solubilized in 2% β-mercaptoethanol and run on 10% uniform gel slabs.

Results and Discussion

As shown in Figure A4.1, networks made in the presence of added 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ were crosslinked by factor XIIIa. Crosslinking was evidenced by γ-dimerization, absence of individual γ chains and α polymerization. All purified fibrin networks made without $CaCl_2$ were not crosslinked and all networks made in plasma were crosslinked.

Figure A4.1 The polyacrylamide gel electrophoresis pattern of crosslinked and non-crosslinked fibrin


EDTA clot
Ca^{++} + Mg^{++} clot
Mg^{++} clot
Control clot
Fibrinogen
Marker Proteins
γ
β
α
γ-γ dimer
α polymers

APPENDIX 5

A MATHEMATICAL TREATMENT OF THE EFFECT OF POLYDISPERSION IN FIBRE DIAMETER ON u_p AND u_T.

The fibrin network is composed of two relatively distinct networks which have been called major and minor networks (Chapter 4). The diameters of the fibrin strands in either network are not uniform, but in the major network they are many times thicker than those in the minor network. The precise distribution of fibres and the differences in their diameters vary depending upon the average mass-length ratio of network fibres. It has been observed that the diameter of fibrin strands in major network in a coarse clot may be up to ten times or more than that in minor network. The difference remains obvious although it is less marked in fine clots.

If for the following discussion it is assumed that the major and minor networks are monodisperse and that diameter of strands in minor network is d and diameter of strands in major network is 10 times d (i.e., 10d) then, presence of the minor network will modify the properties of the clot as follows:

1. Permeation

The permeability or Darcy constant, τ, of a fibrin clot is

given by:

$$\tau = \frac{Q h \eta}{F t p} \qquad \ldots 1$$

where Q is the volume flow through the clot in time t; η is the viscosity of the perfusion fluid, h is the length of the clot; F its cross-section and p is the applied pressure.

The permeability is related to the mass-length ratio, μ, of the fibrin fibres according to the equation:

$$\tau = 4\mu/\pi k c \qquad \ldots 2$$

where c is the concentration of fibrin and k is the packing constant. (Carr et al., 1977)

The mass-length ratio, μ, is given by:

$$\mu = \pi \zeta r^2 \qquad \ldots 3$$

where r is the radius of the fibres and ζ the fibre density.

In this two network model,

$$\mu_{Major} = \pi \xi (10r)^2 = 100\pi\xi r^2 = 100\mu \qquad \ldots 4$$

$$\mu_{Minor} = \pi \xi r^2 = \mu \qquad \ldots 5$$

Furthermore since the fibrin concentration in the minor network is much smaller than that in the major network we assume that

$$C_{Major} = 0.95C \quad \ldots 6$$

$$C_{Minor} = 0.05C \quad \ldots 7$$

Then, substituting these values in equation 2

$$\mu_{Major} = 105.26\tau \quad \ldots 8$$

$$\mu_{Minor} = 20.00\tau \quad \ldots 9$$

The equivalent permeability of a clot composed of more than one network may be defined as the permeability of a homogeneous clot of the same dimensions that would allow equivalent flow rate under identical applied pressure. Depending upon whether the major and minor networks are in series or parallel, the equivalent permeability of the clot will be given by the harmonic or arithmetic mean, respectively (Cardwell and Parsons, 1945).

$$\text{Arithmetic mean} = 1/2 \left(\tau_{Major} + \tau_{Minor} \right) \quad \ldots 10$$

$$\text{Harmonic mean} = \frac{2}{\frac{1}{\tau_{Major}} + \frac{1}{\tau_{Minor}}} \quad \ldots 11$$

Substituting values from equations 3 and 4,

$$\text{Arithmetic mean} = 62.63\tau \quad \ldots 12$$

$$\text{Harmonic mean} = 33.61\tau \quad \ldots 13$$

Since in a clot the two networks are superimposed, the equivalent permeability should correspond to the harmonic

mean. However, according to the theory of fluid dynamics it cannot exceed the arithmetic mean (Cardwell and Parsons, 1945). Thus, the permeability of the clot will be markedly reduced by the minor network and could approach the harmonic mean. Since μ is proportional to τ, the average mass-length ratio of the fibres in the clot will also be lower than that for the major network alone.

2. Turbidity

According to Carr and Hermans (1978), the turbidity of clots made from fibrinogen solutions is given by the equation:

$$T = 44\pi K c \lambda \mu / 15n \qquad \ldots 15$$

where c is the concentration of fibrin fibres, λ wavelength, n is the refractive index of the solution, and μ is as before, the mass-length ratio of the fibres. The constant K is given by

$$K = 2\pi^2 n^2 (dn/dc)^2 / N\lambda^4 \qquad \ldots 16$$

where dn/dc is the specific refractive index increment of the solute in the solvent and N is Avogadro's number. This equation is valid in the limit where the diameter of the fibrin fibres is small compared with the wavelength.

Since the turbidity is proportional to μ, and

$$\mu_{major} = 100\mu_{minor}$$

it follows that all other parameters being equal, the contribution to turbidity from the minor network will be one hundredth that from the major network. Hence, the

contribution from the minor network to the turbidity of the clot may be ignored.

From this mathematical treatment of the methods of measuring μ_p and μ_T it can be seen that the turbidity is influenced more strongly by thicker major network fibres than by the very thin minor network fibres. On the other hand, permeation is especially sensitive to the minor network. Although this mathematical treatment assumes only two superimposed networks, the major network fibre distribution is non-uniform. Thus, even in the absence of minor network, turbidity is sensitive to the thicker fibres in the major network while permeability is more sensitive to its thinner fibre. Consequently, μ_T and μ_p represent the average of different populations of fibres in the fibrin network.

APPENDIX 6

TABULATED DATA OF THE EFFECTS OF CHANGES IN CLOTTING CONDITIONS AND THE EFFECTS OF VARIOUS SUBSTANCES ON FIBRIN NETWORK STRUCTURE AND PROPERTIES

The following tables detail the effect on network stucture of: (1) the thrombin concentraion

(2) fibrinogen concentration

(3) temperature

(4) ionic strength

(5) pH

(6) glucose

(7) protamine sulphate

(8) heparin (1.5 mg/ml fibrinogen)

(9) heparin (3.3 mg/ml fibrinogen)

(10) heparin sulphate

(11) chondroitin-4-sulphate

(12) chondroitin-6-sulphate

(13) citrate

Networks were made earlier in the absence of divalent cations (NDC), in the presence of 2.02 mM $MgCl_2$ (Mg^{++}) or in the presence of 1.2 mM $CaCl_2$ + 0.82 mM $MgCl_2$ (Ca^{++}). ND indicates that the experiment was not done. Data is presented as the mean of at least three determinations $\pm$ SD.

1. THROMBIN	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	lag time (sec)	ΔOD/sec $x10^{-4}$	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
0.05 U/ml								
NDC	37.0 ± 0.5	22.9 ± 0.8	0.690 ± 0.066	9.8 ± 1.0	183 ± 23	5.59 ± 0.29	305 ± 4	2.56 ± 0.02
Ca^{++}	37.7 ± 0.3	24.4 ± 0.2	0.722 ± 0.017	17.0 ± 2.2	108 ± 10	10.7 ± 0.5	307 ± 3	2.60 ± 0.05
0.10 U/ml								
NDC	26.5 ± 0.8	18.6 ± 0.5	0.631 ± 0.056	6.8 ± 0.6	98.6 ± 18.8	11.2 ± 0.7	190 ± 6	2.95 ± 0.01
Ca^{++}	32.9 ± 0.7	22.4 ± 0.2	0.706 ± 0.015	14.0 ± 0.7	62.0 ± 12.0	20.8 ± 1.2	247 ± 5	2.82 ± 0.01
0.15 U/ml								
NDC	23.3 ± 1.4	16.8 ± 0.4	0.596 ± 0.001	6.0 ± 0.8	70.8 ± 6.2	16.7 ± 1.4	161 ± 10	3.06 ± 0.02
Ca^{++}	28.9 ± 1.1	20.5 ± 0.2	0.678 ± 0.014	12.8 ± 2.0	46.8 ± 5.1	28.9 ± 1.3	208 ± 8	2.94 ± 0.02
0.25 U/ml								
NDC	17.8 ± 0.7	15.1 ± 0.2	0.543 ± 0.025	4.2 ± 1.0	44.5 ± 5.8	26.1 ± 0.9	121 ± 5	3.12 ± 0.02
Ca^{++}	23.6 ± 0.6	18.6 ± 0.2	0.638 ± 0.022	9.8 ± 1.8	29.8 ± 3.5	47.1 ± 1.2	164 ± 4	3.05 ± 0.01
0.50 U/ml								
NDC	13.6 ± 0.9	13.3 ± 0.5	0.487 ± 0.013	2.8 ± 0.8	24.3 ± 1.1	49.3 ± 1.8	92 ± 6	3.13 ± 0.01
Ca^{++}	20.1 ± 1.7	16.9 ± 0.3	0.587 ± 0.032	6.7 ± 1.0	17.5 ± 1.9	85.5 ± 2.2	138 ± 12	3.08 ± 0.01

2. FIBRINOGEN	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	lag time (sec)	ΔOD/sec $x10^{-4}$	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
1.0 mg/ml								
NDC	34.2 ± 2.1	19.7 ± 0.3	0.286 ± 0.004	13.8 ± 0.8	30.7 ± 3.1	12.2 ± 0.9	800 ± 51	0.91 ± 0.01
Ca^{++}	28.2 ± 2.1	22.9 ± 0.8	0.306 ± 0.007	28.0 ± 2.2	21.7 ± 0.6	21.0 ± 0.9	657 ± 50	0.91 ± 0.01
2.0 mg/ml								
NDC	25.2 ± 2.2	20.8 ± 0.3	0.436 ± 0.050	9.6 ± 1.9	39.7 ± 3.8	15.6 ± 1.7	299 ± 27	1.79 ± 0.02
Ca^{++}	20.7 ± 0.6	21.6 ± 0.2	0.453 ± 0.006	14.4 ± 1.7	28.7 ± 0.6	25.8 ± 0.4	241 ± 7	1.82 ± 0.01
3.3 mg/ml								
NDC	23.1 ± 1.0	19.7 ± 0.5	0.661 ± 0.013	6.5 ± 1.0	60.3 ± 3.8	20.8 ± 1.1	165 ± 7	2.96 ± 0.02
Ca^{++}	18.1 ± 0.3	17.8 ± 0.2	0.615 ± 0.013	9.3 ± 1.2	38.0 ± 0.6	32.7 ± 1.6	128 ± 2	3.00 ± 0.02
4.0 mg/ml								
NDC	22.7 ± 0.4	18.4 ± 0.3	0.747 ± 0.018	5.5 ± 0.5	62.0 ± 4.0	25.0 ± 1.3	134 ± 3	3.58 ± 0.01
Ca^{++}	28.1 ± 1.0	16.6 ± 0.3	0.685 ± 0.031	8.6 ± 1.4	37.3 ± 0.6	39.0 ± 1.7	107 ± 6	3.59 ± 0.03
6.0 mg/ml								
NDC	22.0 ± 0.5	14.3 ± 0.3	0.890 ± 0.004	3.5 ± 0.5	80.3 ± 1.2	27.3 ± 1.2	5.7 ± 2.1	5.44 ± 0.02
Ca^{++}	13.8 ± 0.8	13.8 ± 0.3	0.860 ± 0.004	6.7 ± 0.6	52.0 ± 0.5	43.6 ± 1.9	53.8 ± 3.1	5.44 ± 0.02
8.0 mg/ml								
NDC	28.6 ± 1.8	12.0 ± 0.3	0.974 ± 0.004	3.5 ± 0.6	103 ± 4.4	29.8 ± 1.4	4.7 ± 5.3	7.16 ± 0.04
Ca^{++}	13.7 ± 1.6	12.3 ± 0.3	0.964 ± 0.005	6.4 ± 0.5	68.7 ± 1.5	42.6 ± 1.2	40.6 ± 4.8	7.17 ± 0.04

3. TEMPERATURE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	lag time (sec)	ΔOD/sec $x10^{-4}$	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
15°C								
NDC	19.8 ± 1.2	7.7 ± 0.1	0.340 ± 0.003	5.6 ± 1.1	66.0 ± 1.0	8.9 ± 0.4	141 ± 9	2.97 ± 0.01
Mg^{++}	33.1 ± 1.4	10.5 ± 0.2	0.382 ± 0.004	14.0 ± 1.3	43.5 ± 5.3	13.7 ± 1.9	259 ± 11	2.70 ± 0.01
Ca^{++}	21.6 ± 1.9	9.7 ± 0.1	0.368 ± 0.001	8.3 ± 0.6	37.8 ± 4.8	14.9 ± 1.4	161 ± 14	2.84 ± 0.03
20°C								
NDC	ND	9.3 ± 0.2	0.361 ± 0.001	ND	52.0 ± 5.3	11.3 ± 1.5	ND	2.96 ± 0.01
Mg^{++}	ND	13.6 ± 0.2	0.438 ± 0.006	ND	43.5 ± 2.1	15.5 ± 0.8	ND	2.68 ± 0.02
Ca^{++}	ND	10.3 ± 0.1	0.385 ± 0.004	ND	35.8 ± 4.1	17.7 ± 1.4	ND	2.85 ± 0.02
22°C								
NDC	23.6 ± 0.5	10.6 ± 0.4	0.398 ± 0.002	4.6 ± 0.9	54.3 ± 2.5	11.5 ± 5.5	169 ± 4	2.95 ± 0.01
Mg^{++}	36.7 ± 0.5	12.8 ± 0.5	0.425 ± 0.015	14.0 ± 1.0	45.0 ± 7.0	14.0 ± 2.9	290 ± 4	2.68 ± 0.03
Ca^{++}	18.7 ± 0.8	10.7 ± 0.2	0.397 ± 0.003	8.2 ± 0.3	36.4 ± 4.5	19.0 ± 0.9	138 ± 6	2.87 ± 0.02
25°C								
NDC	ND	11.5 ± 0.2	0.411 ± 0.004	ND	44.0 ± 1.7	16.3 ± 0.5	ND	2.98 ± 0.01
Mg^{++}	ND	14.6 ± 0.3	0.466 ± 0.011	ND	35.0 ± 2.9	21.3 ± 1.3	ND	2.73 ± 0.01
Ca^{++}	ND	11.9 ± 0.1	0.426 ± 0.003	ND	35.4 ± 1.9	20.0 ± 1.2	ND	2.90 ± 0.03

3. TEMP. Contd.	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	lag time (sec)	ΔOD/sec $x10^{-4}$	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
30°C								
NDC	26.2 ± 3.9	12.8 ± 0.1	0.447 ± 0.007	6.0 ± 0.9	48.3 ± 2.1	14.9 ± 0.9	186 ± 28	2.99 ± 0.01
Mg^{++}	43.5 ± 1.2	16.8 ± 0.5	0.550 ± 0.019	14.7 ± 0.6	42.7 ± 1.5	17.7 ± 1.1	331 ± 9	2.78 ± 0.03
Ca^{++}	21.4 ± 1.5	12.8 ± 0.2	0.421 ± 0.013	9.0 ± 1.0	37.0 ± 1.9	21.6 ± 0.9	154 ± 11	2.94 ± 0.01
35°C								
NDC	ND	14.6 ± 0.1	0.500 ± 0.004	ND	49.3 ± 2.3	14.4 ± 0.6	ND	3.00 ± 0.02
Mg^{++}	ND	17.9 ± 0.1	0.572 ± 0.008	ND	36.3 ± 1.5	21.6 ± 0.5	ND	2.80 ± 0.01
Ca^{++}	ND	14.1 ± 0.3	0.485 ± 0.007	ND	36.0 ± 1.7	23.7 ± 0.6	ND	2.99 ± 0.01
37°C								
NDC	34.4 ± 1.6	15.1 ± 0.4	0.520 ± 0.016	7.8 ± 0.6	54.7 ± 3.8	12.6 ± 0.3	240 ± 11	3.03 ± 0.01
Mg^{++}	56.0 ± 1.3	18.2 ± 0.2	0.586 ± 0.004	16.0 ± 1.0	43.3 ± 4.0	18.9 ± 0.9	418 ± 10	2.84 ± 0.02
Ca^{++}	23.7 ± 2.1	15.7 ± 0.3	0.492 ± 0.004	9.2 ± 1.3	46.3 ± 5.0	21.2 ± 1.8	167 ± 15	3.01 ± 0.01
40°C								
NDC	38.4 ± 3.4	18.2 ± 0.2	0.614 ± 0.001	8.5 ± 0.5	72.0 ± 4.4	12.9 ± 0.9	271 ± 24	3.00 ± 0.02
Mg^{++}	61.2 ± 2.3	21.4 ± 0.3	0.653 ± 0.010	18.0 ± 0.5	58.0 ± 3.0	18.4 ± 0.1	479 ± 18	2.70 ± 0.06
Ca^{++}	31.0 ± 2.8	17.8 ± 0.3	0.585 ± 0.007	10.0 ± 1.0	50.5 ± 3.5	24.4 ± 2.2	222 ± 20	2.96 ± 0.02

4. IONIC STRENGTH	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	lag time (sec)	ΔOD/sec $x10^{-4}$	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
0.133								
NDC	29.3 ± 0.9	13.3 ± 0.4	0.448 ± 0.010	8.3 ± 1.6	103 ± 9	38.1 ± 2.1	228 ± 7	2.90 ± 0.02
Mg^{++}	42.3 ± 1.7	16.1 ± 0.6	0.490 ± 0.014	16.0 ± 2.4	123 ± 7	46.9 ± 4.3	326 ± 14	2.66 ± 0.05
Ca^{++}	13.0 ± 0.6	14.7 ± 0.1	0.451 ± 0.014	9.9 ± 3.1	80 ± 7	52.8 ± 4.5	95 ± 4	2.79 ± 0.05
0.143								
NDC	21.7 ± 0.5	13.3 ± 0.6	0.441 ± 0.017	6.5 ± 1.3	132 ± 10	34.7 ± 2.6	157 ± 4	2.87 ± 0.03
Mg^{++}	29.4 ± 1.9	16.5 ± 0.6	0.495 ± 0.012	11.9 ± 1.6	127 ± 7	39.5 ± 5.8	216 ± 14	2.64 ± 0.02
Ca^{++}	9.2 ± 1.2	14.9 ± 0.7	0.467 ± 0.020	8.0 ± 1.5	91 ± 4	46.0 ± 3.0	65.1 ± 8.3	2.80 ± 0.04
0.153								
NDC	14.2 ± 1.6	12.3 ± 0.4	0.426 ± 0.011	4.8 ± 0.7	120 ± 12	30.4 ± 2.6	99.3 ± 11.3	2.98 ± 0.02
Mg^{++}	20.7 ± 1.9	15.2 ± 0.6	0.463 ± 0.009	7.0 ± 0.6	161 ± 7	34.6 ± 5.2	148 ± 14	2.70 ± 0.02
Ca^{++}	6.8 ± 1.3	13.5 ± 0.1	0.444 ± 0.018	7.5 ± 1.4	106 ± 8	42.6 ± 1.5	47.4 ± 9.2	2.81 ± 0.03
0.163								
NDC	2.72 ± 0.6	11.4 ± 0.4	0.405 ± 0.010	3.2 ± 0.7	144 ± 14	22.3 ± 2.1	18.8 ± 4.2	3.02 ± 0.03
Mg^{++}	12.3 ± 1.2	14.2 ± 0.2	0.453 ± 0.006	5.1 ± 0.8	144 ± 21	28.5 ± 3.8	870 ± 8.8	2.78 ± 0.02
Ca^{++}	4.0 ± 0.5	12.7 ± 0.3	0.422 ± 0.007	6.5 ± 0.8	141 ± 12	37.9 ± 4.4	27.0 ± 3.3	2.89 ± 0.03
0.173								
NDC	0.64 ± 0.04	9.7 ± 0.2	0.356 ± 0.009	2.0 ± 0.4	171 ± 19.4	17.7 ± 0.4	4.48 ± 0.26	3.02 ± 0.05
Mg^{++}	1.55 ± 0.02	12.2 ± 0.4	0.400 ± 0.010	3.3 ± 0.7	169 ± 28	20.8 ± 1.3	11.1 ± 1.5	2.79 ± 0.02
Ca^{++}	2.11 ± 0.2	10.7 ± 0.3	0.365 ± 0.009	5.3 ± 0.7	118 ± 10	36.3 ± 3.3	14.3 ± 1.8	2.89 ± 0.03

5. pH		$\mu_p x 10^{12}$ (Daltons/cm)	$\mu_T x 10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
7.15							
	NDC	33.7 ± 2.0	11.0 ± 0.4	0.427 ± 0.011	7.0 ± 0.8	247 ± 14	2.90 ± 0.01
	Mg^{++}	58.7 ± 4.0	15.6 ± 0.3	0.532 ± 0.008	15.5 ± 2.9	466 ± 32	2.66 ± 0.03
	Ca^{++}	22.3 ± 2.6	13.5 ± 0.5	0.485 ± 0.019	11.6 ± 2.3	176 ± 20	2.79 ± 0.02
7.25							
	NDC	30.3 ± 2.6	10.7 ± 0.2	0.417 ± 0.009	6.3 ± 1.0	223 ± 19	2.87 ± 0.04
	Mg^{++}	53.0 ± 4.1	14.9 ± 0.4	0.505 ± 0.010	15.1 ± 1.7	424 ± 32	2.64 ± 0.04
	Ca^{++}	27.1 ± 0.6	12.4 ± 0.7	0.453 ± 0.016	9.4 ± 1.5	205 ± 4	2.80 ± 0.04
7.35							
	NDC	25.4 ± 1.2	9.6 ± 0.3	0.391 ± 0.009	5.0 ± 1.1	179 ± 8	2.98 ± 0.01
	Mg^{++}	42.9 ± 1.5	13.6 ± 0.4	0.479 ± 0.014	12.5 ± 1.4	336 ± 14	2.70 ± 0.04
	Ca^{++}	22.1 ± 1.4	11.7 ± 0.3	0.433 ± 0.009	8.8 ± 1.3	166 ± 10	2.81 ± 0.03
7.45							
	NDC	21.2 ± 2.3	9.2 ± 0.2	0.385 ± 0.007	4.0 ± 0.6	148 ± 16	3.02 ± 0.03
	Mg^{++}	41.1 ± 1.9	12.5 ± 0.3	0.459 ± 0.008	10.7 ± 1.8	312 ± 14	2.78 ± 0.04
	Ca^{++}	18.6 ± 0.5	10.9 ± 0.3	0.417 ± 0.007	7.7 ± 1.1	137 ± 4	2.89 ± 0.04
7.55							
	NDC	17.9 ± 1.2	9.2 ± 0.3	0.380 ± 0.010	3.4 ± 0.4	125 ± 8	3.02 ± 0.02
	Mg^{++}	32.9 ± 1.8	11.2 ± 0.4	0.426 ± 0.011	9.1 ± 0.2	249 ± 14	2.79 ± 0.02
	Ca^{++}	15.8 ± 1.7	11.0 ± 0.4	0.419 ± 0.012	6.6 ± 1.2	116 ± 13	2.89 ± 0.05

6. GLUCOSE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	13.3 ± 1.7	18.5 ± 0.2	0.617 ± 0.035	2.9 ± 0.7	94.7 ± 12.2	2.95 ± 0.09
Ca^{++}	16.2 ± 2.3	25.7 ± 0.4	0.786 ± 0.049	6.0 ± 0.4	114 ± 16	3.01 ± 0.02
10 mM						
NDC	9.7 ± 1.8	17.6 ± 1.4	0.580 ± 0.023	2.0 ± 0.6	71.8 ± 13.6	2.85 ± 0.03
Ca^{++}	11.0 ± 0.5	24.7 ± 0.8	0.745 ± 0.015	6.5 ± 0.5	80.6 ± 4.0	2.87 ± 0.05
40 mM						
NDC	4.53 + 0.83	15.3 + 0.3	0.463 + 0.012	<2.0	39.2 + 7.2	2.44 + 0.09
Ca^{++}	6.92 ± 0.56	21.3 ± 0.5	0.702 ± 0.048	5.6 ± 0.5	56.8 ± 4.6	2.58 ± 0.01
70 mM						
NDC	0.80 + 0.14	10.4 + 0.8	0.355 + 0.013	<2.0	5.73 + 1.40	2.13 ± 0.08
Ca^{++}	4.58 ± 0.52	14.4 ± 0.5	0.699 ± 0.057	4.2 ± 0.4	45.1 ± 5.2	2.15 ± 0.12
100 mM						
NDC	0.41 + 0.03	10.7 ± 1.3	0.314 ± 0.002	<2.0	6.0 ± 0.5	1.40 ± 0.11
Ca^{++}	1.53 ± 0.49	13.9 ± 0.4	0.566 ± 0.018	2.8 ± 0.4	22.1 ± 7.2	1.46 ± 0.10

7. PROTAMINE SULPHATE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	22.5 ± 2.1	14.1 ± 0.2	0.287 ± 0.001	6.8 ± 1.3	349 ± 33	1.36 ± 0.02
Ca^{++}	19.0 ± 3.9	19.9 ± 0.2	0.348 ± 0.008	10.2 ± 1.9	305 ± 63	1.32 ± 0.03
0.1 µg/ml						
NDC	31.5 ± 1.1	14.2 ± 0.3	0.289 ± 0.007	9.6 ± 0.9	489 ± 17	1.36 ± 0.01
Ca^{++}	33.4 ± 2.8	21.7 ± 0.6	0.355 ± 0.003	14.4 ± 1.8	536 ± 45	1.33 ± 0.02
0.25 µg/ml						
NDC	35.7 ± 1.8	17.6 ± 1.5	0.316 ± 0.008	10.3 ± 2.1	560 ± 28	1.35 ± 0.01
Ca^{++}	57.9 ± 4.5	23.2 ± 0.4	0.369 ± 0.006	17.0 ± 2.2	912 ± 71	1.34 ± 0.01
0.5 µg/ml						
NDC	57.4 ± 1.9	21.9 ± 0.7	0.353 ± 0.008	12.6 ± 2.3	917 ± 30	1.33 ± 0.02
Ca^{++}	68.6 ± 5.0	30.9 ± 0.6	0.465 ± 0.007	25.2 ± 3.2	1086 ± 79	1.34 ± 0.01
0.75 µg/ml						
NDC	77.7 ± 1.5	29.6 ± 0.7	0.455 ± 0.009	15.0 ± 2.2	222 ± 24	1.34 ± 0.01
Ca^{++}	80.1 ± 3.6	35.3 ± 1.0	0.521 ± 0.016	29.7 ± 3.0	1271 ± 57	1.33 ± 0.02
1.0 µg/ml						
NDC	105.4 ± 11.0	31.2 ± 3.0	0.507 ± 0.017	19.6 ± 4.4	666 ± 174	1.34 ± 0.02
Ca^{++}	91.6 ± 3.9	34.4 ± 0.8	0.514 ± 0.011	40.3 ± 5.0	1422 ± 61	1.36 ± 0.01

8. HEPARIN	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	22.5 ± 2.1	17.4 ± 2.2	0.287 ± 0.001	6.8 ± 1.3	349 ± 33	1.36 ± 0.02
Ca^{++}	19.0 ± 3.9	21.4 ± 1.8	0.383 ± 0.008	10.2 ± 1.9	305 ± 63	1.32 ± 0.03
0.18 U/ml						
NDC	22.2 ± 1.5	12.2 ± 0.1	0.263 ± 0.003	9.1 ± 2.1	357 ± 24	1.32 ± 0.02
Ca^{++}	18.6 ± 1.8	18.6 ± 0.3	0.300 ± 0.002	9.1 ± 1.3	304 ± 3	1.29 ± 0.03
0.50 U/ml						
NDC	ND	9.4 ± 4.0	0.268 ± 0.005	ND	ND	1.28 ± 0.04
Ca^{++}	ND	7.3 ± 0.1	0.288 ± 0.002	ND	ND	1.29 ± 0.04
0.90 U/ml						
NDC	21.9 ± 1.0	15.9 ± 0.3	0.281 ± 0.003	11.5 ± 1.6	371 ± 17	1.25 ± 0.03
Ca^{++}	19.4 ± 2.1	18.8 ± 0.1	0.305 ± 0.001	11.2 ± 1.4	321 ± 35	1.28 ± 0.02
1.8 U/ml						
NDC	29.2 ± 1.0	19.0 ± 0.6	0.310 ± 0.008	13.5 ± 2.2	485 ± 17	1.27 ± 0.04
Ca^{++}	20.6 ± 0.7	22.4 ± 0.40	0.336 ± 0.006	13.3 ± 2.1	347 ± 11	1.26 ± 0.03
9.0 U/ml						
NDC	39.0 ± 2.0	24.2 ± 0.3	0.387 ± 0.006	24.0 ± 1.7	604 ± 31	1.36 ± 0.01
Ca^{++}	28.6 ± 2.3	27.8 ± 0.2	0.419 ± 0.006	20.7 ± 1.5	449 ± 18	1.35 ± 0.01
18.0 U/ml						
NDC	74.6 ± 7.7	32.9 ± 0.1	0.501 ± 0.005	29.0 ± 2.0	143 ± 118	1.38 ± 0.01
Ca^{++}	41.9 ± 1.3	34.3 ± 0.4	0.520 ± 0.005	29.2 ± 3.5	646 ± 20	1.37 ± 0.01

9. HEPARIN (3.3 mg/ml)	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	18.8 ± 1.4	17.3 ± 0.5	0.601 ± 0.018	5.5 ± 2.3	130 ± 10	3.06 ± 0.01
Ca^{++}	15.6 ± 1.0	19.9 ± 0.2	0.740 ± 0.008	9.5 ± 1.5	109 ± 7	3.02 ± 0.03
0.18 U/ml						
NDC	19.9 ± 1.1	12.6 ± 0.5	0.436 ± 0.012	5.3 ± 1.5	140 ± 8	3.00 ± 0.01
Ca^{++}	19.2 ± 1.5	17.8 ± 1.1	0.576 ± 0.027	8.9 ± 0.9	138 ± 11	2.94 ± 0.03
0.50 U/ml						
NDC	ND	9.8 ± 0.3	0.361 ± 0.009	ND	ND	2.90 ± 0.03
Ca^{++}	ND	16.1 ± 0.4	0.519 ± 0.005	ND	ND	2.92 ± 0.04
0.90 U/ml						
NDC	17.4 ± 0.3	12.5 ± 0.1	0.415 ± 0.007	6.5 ± 1.0	126 ± 2	2.92 ± 0.03
Ca^{++}	17.0 ± 2.9	17.9 ± 0.4	0.562 ± 0.008	9.0 ± 1.2	124 ± 21	2.91 ± 0.02
1.8 U/ml						
NDC	19.7 ± 2.5	16.6 ± 0.3	0.530 ± 0.011	11.3 ± 3.5	143 ± 18	2.91 ± 0.01
Ca^{++}	16.9 ± 2.8	19.3 ± 0.2	0.612 ± 0.007	10.0 ± 2.6	121 ± 20	2.95 ± 0.02
9.0 U/ml						
NDC	38.6 ± 2.7	25.4 ± 1.2	0.814 ± 0.034	19.8 ± 4.9	273 ± 19	2.99 ± 0.03
Ca^{++}	30.1 ± 2.6	27.1 ± 0.3	0.876 ± 0.006	18.8 ± 2.9	212 ± 18	3.00 ± 0.02
18.0 U/ml						
NDC	46.2 ± 4.4	28.4 ± 0.2	0.908 ± 0.005	26.3 ± 2.5	323 ± 31	3.02 ± 0.03
Ca^{++}	32.4 ± 2.3	17.6 ± 5.0	0.926 ± 0.006	22.3 ± 4.7	224 ± 16	3.06 ± 0.02

10. HEPARIN SULPHATE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	ND	ND	0.287 ± 0.003	6.8 ± 1.3	349 ± 33	ND
Ca^{++}	ND	ND	0.348 ± 0.008	8.5 ± 2.8	305 ± 63	ND
0.1 mg/ml						
NDC	ND	ND	0.289 ± 0.003	8.9 ± 1.9	407 ± 30	ND
Ca^{++}	ND	ND	0.338 ± 0.005	12.3 ± 1.6	445 ± 56	ND
0.28 mg/ml						
NDC	ND	ND	0.300 ± 0.007	8.3 ± 1.9	352 ± 18	ND
Ca^{++}	ND	ND	0.354 ± 0.002	14.0 ± 2.0	443 ± 28	ND
0.5 mg/ml						
NDC	ND	ND	0.318 ± 0.003	11.0 ± 1.1	401 ± 20	ND
Ca^{++}	ND	ND	0.372 ± 0.003	15.7 ± 1.6	447 ± 35	ND
1.0 mg/ml						
NDC	ND	ND	0.348 ± 0.005	13.3 ± 1.9	452 ± 4	ND
Ca^{++}	ND	ND	0.404 ± 0.004	18.0 ± 3.4	561 ± 62	ND
10 mg/ml						
NDC	ND	ND	0.437 ± 0.005	27.2 ± 1.7	605 ± 15	ND
Ca^{++}	ND	ND	0.534 ± 0.011	36.5 ± 5.2	958 ± 49	ND

11. CHONDROITIN-4 SULPHATE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	13.3 ± 0.8	20.3 ± 1.5	0.325 ± 0.003	6.4 ± 0.8	227 ± 13	3.04 ± 0.04
Ca^{++}	16.1 ± 1.5	26.9 ± 0.5	0.389 ± 0.010	14.0 ± 1.3	265 ± 25	3.08 ± 0.02
35 µg/ml						
NDC	18.9 ± 0.4	26.6 ± 0.8	0.344 ± 0.007	7.8 ± 0.9	318 ± 7	3.05 ± 0.02
Ca^{++}	19.6 ± 0.9	27.4 ± 1.1	0.403 ± 0.002	13.7 ± 1.5	317 ± 15	3.11 ± 0.02
70 µg/ml						
NDC	19.7 ± 0.8	26.1 ± 1.2	0.375 ± 0.005	8.5 ± 1.0	328 ± 14	3.07 ± 0.04
Ca^{++}	20.3 ± 0.4	29.6 ± 1.4	0.436 ± 0.009	14.0 ± 1.0	327 ± 6	3.11 ± 0.01
350 µg/ml						
NDC	26.2 ± 1.1	33.6 ± 1.2	0.481 ± 0.002	13.3 ± 0.7	425 ± 18	3.10 ± 0.01
Ca^{++}	25.3 ± 0.5	36.8 ± 1.2	0.538 ± 0.004	17.3 ± 1.8	399 ± 8	3.14 ± 0.01
700 µg/ml						
NDC	34.3 ± 2.6	41.3 ± 1.6	0.583 ± 0.008	18.2 ± 4.8	557 ± 43	3.10 ± 0.03
Ca^{++}	26.3 ± 0.8	41.6 ± 1.3	0.608 ± 0.006	25.7 ± 1.2	411 ± 12	3.15 ± 0.02

12. CHONDROITIN-6 SULPHATE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	13.3 ± 0.8	20.3 ± 1.5	0.325 ± 0.003	6.4 ± 0.8	227 ± 13	3.04 ± 0.04
Ca^{++}	16.1 ± 1.5	26.9 ± 0.5	0.398 ± 0.004	14.0 ± 1.3	265 ± 25	3.08 ± 0.04
35 µg/ml						
NDC	18.9 ± 2.1	26.5 ± 1.0	0.376 ± 0.001	9.0 ± 1.2	317 ± 35	3.06 ± 0.05
Ca^{++}	18.6 ± 0.7	27.5 ± 1.0	0.398 ± 0.001	12.7 ± 1.2	306 ± 12	3.09 ± 0.03
70 µg/ml						
NDC	22.3 ± 1.7	27.2 ± 1.0	0.397 ± 0.009	8.4 ± 0.6	368 ± 28	3.08 ± 0.04
Ca^{++}	19.6 ± 0.6	29.8 ± 1.5	0.430 ± 0.006	14.3 ± 1.1	320 ± 9	3.10 ± 0.01
350 µg/ml						
NDC	35.2 ± 0.9	39.0 ± 1.0	0.558 ± 0.013	16.1 ± 1.4	571 ± 14	3.10 ± 0.04
Ca^{++}	29.6 ± 1.8	39.0 ± 0.6	0.575 ± 0.010	17.7 ± 1.2	464 ± 28	3.15 ± 0.01
700 µg/ml						
NDC	51.4 ± 3.7	45.5 ± 1.2	0.658 ± 0.003	24.8 ± 1.3	821 ± 59	3.12 ± 0.01
Ca^{++}	36.3 ± 3.6	42.9 ± 0.8	0.622 ± 0.002	23.0 ± 2.8	574 ± 57	3.14 ± 0.02

13. CITRATE	$\mu_p x10^{12}$ (Daltons/cm)	$\mu_T x10^{12}$ (Daltons/cm)	opacity ratio	compaction (%)	Darcy Const. $x10^{11}$ (cm^2)	Fibrin conc. (mg/ml)
Control						
NDC	13.6 ± 1.8	18.2 ± 1.4	0.617 ± 0.035	2.9 ± 0.7	94.7 ± 12.2	3.04 ± 0.02
Ca^{++}	8.7 ± 0.5	20.2 ± 1.2	0.693 ± 0.040	6.0 ± 0.4	59.3 ± 3.2	3.10 ± 0.02
2.5 mM						
NDC	13.3 ± 2.6	18.9 ± 1.0	0.640 ± 0.031	3.2 ± 0.3	92.7 ± 18.0	3.03 ± 0.01
Ca^{++}	9.4 ± 0.4	21.4 ± 2.4	0.710 ± 0.029	6.6 ± 1.1	65.5 ± 2.7	3.05 ± 0.02
5.0 mM						
NDC	15.0 ± 1.3	23.0 ± 0.4	0.729 ± 0.061	4.0 ± 1.0	105 ± 9	3 03 ± 0.01
Ca^{++}	13.8 ± 1.7	23.1 ± 0.8	0.776 ± 0.017	7.0 ± 0.5	95.8 ± 12.0	3.04 ± 0.02
7.5 mM						
NDC	19.3 ± 3.3	22.9 ± 1.2	0.760 ± 0.032	5.8 ± 0.8	135 ± 23	3.02 ± 0.02
Ca^{++}	20.8 ± 2.2	24.3 ± 1.2	0.812 ± 0.038	7.0 ± 0.4	133 ± 15	3.06 ± 0.03
10.0 mM						
NDC	24.5 ± 4.2	23.6 ± 1.1	0.795 ± 0.041	6.7 ± 0.5	170 ± 29	3.04 ± 0.01
Ca^{++}	21.8 ± 2.0	24.9 ± 0.7	0.827 ± 0.033	7.4 ± 0.6	152 ± 28	3.03 ± 0.03
12.9 mM						
NDC	23.5 ± 3.4	24.6 ± 1.2	0.794 ± 0.034	9.8 ± 1.8	166 ± 24	2.99 ± 0.03
Ca^{++}	23.5 ± 3.9	25.2 ± 1.2	0.843 ± 0.031	9.3 ± 1.5	163 ± 27	3.04 ± 0.01